Microsatellite variation in the Siberian flying squirrel in Finland

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The Siberian flying squirrel (*Pteromys volans*) is an arboreal rodent that inhabits spruce-dominated boreal forest. The species has declined in Finland, probably due to modern forestry. We studied genetic variation in the flying squirrel ($n = 159$) between eight sampling sites in Finland using seven microsatellite loci. Heterozygosity levels were similar in the flying squirrel as found for other Sciurid species. There were slight heterozygote excesses over all loci. Isolation by distance was clear and genetic differentiation between sampling sites was high (overall $F_{ST} = 0.115$). In addition, geographical isolation of one site was observed as increased differentiation and low allele richness. Radio telemetry studies have revealed that the dispersal abilities of flying squirrels are good. However, the high differentiation between sampling sites indicates that the actual gene flow over large distances is low. Thus our results emphasize the importance of landscape-level management in the conservation of the flying squirrel.

Introduction

Due to their high variability nuclear DNA microsatellite markers are useful for studies of population differentiation (Goldstein & Schlötterer 1999). For endangered or threatened species data of genetic population structure can be essential information for the development of appropriate conservation guidelines (Beaumont & Bruford 1999). The Siberian flying squirrel (*Pteromys volans*) inhabits Eurasian spruce-dominated boreal forests, where it requires deciduous trees as a food resource and tree cavities for roosting and nesting. The flying squirrel has declined in Finland during the last decades probably due to modern forestry (Hokkanen *et al*. 1982, Hanski *et al*. 2001). Based on census data there was a linear decline of the Finnish population to half the original census size between 1950 and 1980 (Hokkanen *et al*. 1982). During the last decade the population seems to have decreased by about 30% (Hanski *et al*. 2001). Intensive felling of forests over large areas in Finland has occurred in the decades since the 1950s, which is probably behind the recent flying squirrel decline. In managed forests the lack of big aspens, providing nest cavities, and a general lack of deciduous trees as food resources, also decreases the amount of breeding habitat for flying squirrels (Selonen & Hanski 2004).

The flying squirrel is a nocturnal, arboreal rodent. The mating system is promiscuous, with males having much larger home ranges than females and male home ranges overlapping those

of several females (Ihalempiä 2000, Hanski *et al.* 2001). Natal dispersal distances of flying squirrels are high, on average 2.5 km, but can be up to 9 km. During dispersal individuals are able to cross very heterogeneous areas and can use low-quality forests for temporary roosting sites (Selonen & Hanski 2004). Due to good dispersal abilities it seems that the landscape of southern Finland is structurally continuous for flying squirrels (Selonen & Hanski 2003, 2004). Thus, although wide open areas restrict dispersal of flying squirrels, gene flow over large distances in southern Finland should be possible.

However, the densities of flying squirrels in Finland are low (Hanski *et al.* 2001), as compared with, for example, those in North American flying squirrels (*Glaucomys volans*, Gilmore & Gates 1985, *Glaucomys sabrinus*, Carey *et al.* 1997). Good quality habitat patches are scarce in the landscape and consequently many of the dispersers end up in habitats that likely function as habitat sinks (Selonen & Hanski 2004). This may increase the genetic differentiation by preventing gene flow between sites.

Although we have detailed data on dispersal distances and the proportions of dispersing flying squirrels (Selonen & Hanski 2004), the level of migration over a large scale is still unknown. By estimating levels of genetic differentiation between sampling sites we can get indirect information on gene flow over a wider scale than with direct methods (Slatkin 1994). It is necessary, however, to be cautious when making interpretations from genetic structure to actual movement (Neigel 2002). Here we used seven microsatellite loci to study the degree of genetic differentiation and isolation of the flying

squirrel, in a situation where dispersal abilities of individuals are good but good quality habitat patches are scarce in the landscape and the densities of individuals are low.

Material and methods

Data collection and study areas

Hair samples were taken from adults ($n = 159$) captured in roosting nests from eight different sampling sites (*see* Table 1 and Fig. 1 for the sampling sites and sample sizes). Samples were taken from one individual per nest (sometimes there may be a few adults in the same nest, although usually only one adult is found per nest). The sex ratio of the five southern sites is approximately 1:1 (I. K. Hanski & V. Selonen unpubl. data), but the three western samples were not sexed. Juveniles were excluded, thus the data reflect post-dispersal sampling. We have also used our data for parental analysis (I. K. Hanski *et al.* unpubl. data), in which we found no indication for null alleles or allelic dropout, which would have increased the heterozygote deficit.

The southern sites were located between 2 to 130 km apart and the western sites, 300 km northwest from the southern sites, between 90 to 135 km apart. Anjalankoski W and E were in practice the same area, but in order to describe the distribution of genetic variation over a small geographic scale the Anjalankoski area was divided into two parts separated by two kilometres. The sizes of sampling sites were 2 km² in AW and 8.5 km² in AE, 0.62 km² in I, 35.8 km² in K, 12 km² in N, 25 km² in L, 5 km² in M, and

Table 1. Measures of genetic diversity among eight flying squirrel sampling sites by seven microsatellite loci. F_{IS} test for heterozygote excess or deficiency (NS, $p > 0.05$, * $p < 0.05$).

Site	N	H_E ± S.E.	H_0 ± S.E.	F_{IS}	No. of alleles ± S.E.	Allelic richness ± S.E.
Iitti	24	0.606 ± 0.084	0.634 ± 0.041	−0.048 [NS]	5.43 ± 2.07	3.33 ± 1.05
Kuusankoski	30	0.701 ± 0.056	0.705 ± 0.035	0.002 [NS]	5.86 ± 1.77	3.70 ± 1.01
Nuuksio	18	0.748 ± 0.032	0.755 ± 0.042	−0.009 [NS]	6.57 ± 1.51	4.09 ± 0.66
Anjalankoski E	42	0.724 ± 0.051	0.772 ± 0.025	−0.067*	7.14 ± 3.08	3.93 ± 1.08
Anjalankoski W	10	0.698 ± 0.059	0.653 ± 0.059	0.068 [NS]	4.86 ± 1.21	3.73 ± 0.87
Alavus	15	0.586 ± 0.075	0.603 ± 0.049	−0.032 [NS]	4.57 ± 1.40	3.12 ± 0.88
Mustasaari	8	0.654 ± 0.066	0.561 ± 0.070	0.149 [NS]	4.00 ± 1.83	3.48 ± 1.73
Luoto	12	0.534 ± 0.040	0.587 ± 0.057	−0.104 [NS]	3.00 ± 1.00	2.50 ± 0.51

27 km^2 in Al (these sizes cover a smaller area than is the maximum dispersal distance).

DNA extraction and microsatellites

DNA was extracted from approximately 20 hairs per individual by cutting 1 cm from the root end of the hair and placing it in 200 μL of 5% Chelex solution. The samples were incubated at 56 °C for 1 h with mixing every ten minutes and then at > 90 °C for five minutes. Two μl of the supernatant was added as a template in the PCR. Each 10-μL PCR mixture contained 1 × PCR buffer plus NHSO$_4$ (MBI Fermentas), 1.5 μM MgCL$_2$, 0.1 mM dNTPs, 1 μl BSA, 10 pmol of unlabelled primer, 1 pmol of fluorescently labelled primer and 0.5 U *Taq* polymerase. The PCR amplification comprised initial denaturation of 2 min at 92 °C, followed by 30 cycles of 30 s at 92 °C, 30 s at 50 or 55 °C, and 30 s at 72 °C, and in the end 10 min at 72 °C. The PCR products were run on an ABI Prism 377 automated sequencer (Applied Biosystems). Details of the PCR protocols and the cloning and characterisation of the microsatellite loci are given in Painter *et al.* (2004). Seven loci were found useful for this study (Pvol10, Pvol41, Pvol74, PvolE1, PvolE5, PvolE6, PvolE10). The Siberian flying squirrel is relatively distantly related to other squirrel species and loci designed for other squirrel species were not successfully amplified for the Siberian flying squirrel (Painter *et al.* 2004).

Statistics

The observed (H_0) and expected (H_E) Hardy-Weinberg gene diversity and the number of alleles per locus were estimated with GENEPOP 3.1 (Raymond & Rousset 1995). Allelic richness, i.e. the number of alleles independent of sample size, was estimated using FSTAT 2.9.3. (Goudet 2001). The allelic richness and H_E were compared among sites using Kruskal-Wallis analysis of variance with different loci as independent observations (Archie 1985).

Deviations from the Hardy-Weinberg equilibrium across loci and sites were tested with the score test in GENEPOP using the Markov
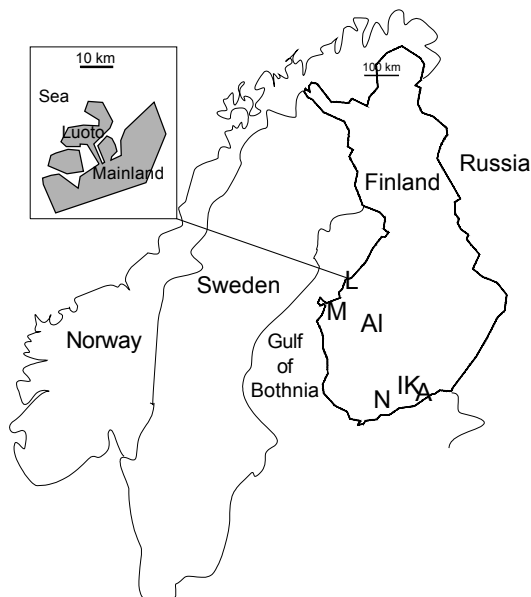
Fig. 1. Flying squirrel sampling sites in Finland. I = Iitti, K = Kuusankoski, N = Nuuksio, A = Anjalankoski (Anjalankoski W and E), Al = Alavus, M = Mustasaari, L = Luoto. Dark raster indicates the land area in the peninsula of Luoto.

chain method to avoid biases due to rare alleles and small sample sizes (Raymond & Rousset 1995). Global deviations from H-W equilibrium were tested by determining F_{IS} according to Weir and Cockerham (1984) using FSTAT. Confidence limits were obtained by bootstrapping and jackknifing. Linkage disequilibrium was tested by Fisher's exact test with the Markov chain method using GENEPOP. Multiple tests were corrected by Bonferroni correction (Rice 1989).

Differentiation between sampling sites was studied using exact probability tests, with Fisher's combined probability method (Sokal & Rolf 1995) for differences in allelic frequencies among sites, and by estimating overall and pairwise F_{ST}. F_{ST} was estimated according to Weir and Cockerham (1984). Confidence limits were obtained by 2000 bootstrapping replicates (using FSTAT).

Isolation by distance was studied with a simple Mantel test (Mantel 1967) between data matrices of pairwise F_{ST} and geographical distance. In addition, the effect of isolation was studied for the Luoto (Eugmo) study area, which

is situated in the peninsula on the coast of the Gulf of Bothnia, connected to the mainland by a forested land bridge less than 1 km wide (Fig. 1). Thus the Luoto population may be isolated from the mainland. Whether Luoto was more differentiated than the sites in the mainland was tested with a partial Mantel test, testing for an association between pairwise F_{ST} comparisons with Luoto versus the comparisons without Luoto, after controlling for the effect of geographical distance (using the program ZT, Bonnet & Van de Peer 2002).

Results

All seven microsatellite loci were polymorphic with between 6–14 alleles. Mean H_0 and H_E varied between 0.56–0.77 and 0.53–0.75, respectively (Table 1). Allelic richness differed between

sites (Kruskal-Wallis: $H_7 = 14$, $p = 0.05$, Tukey: $p < 0.05$ between Luoto and Nuuksio), but H_E did not differ between sites ($H_7 = 12$, $p = 0.10$).

Deviations from Hardy-Weinberg equilibrium were not found for loci/site and site/loci tests ($p > 0.05$, after Bonferroni correction). However, global tests indicated that heterozygote excess occurred both within sites (one site, Table 1) and within loci (overall loci $F_{IS} = -0.023$, Table 2). Only one individual per nest was sampled to avoid sampling of relatives and there was no indication of null alleles or allelic dropout (*see* Material and methods). There was no indication of linkage disequilibrium between loci (all $p > 0.05$, after Bonferroni corrections).

Based on F_{ST} values the differentiation between sites was significant for each locus and overall (overall $F_{ST} = 0.115$, 99% c.l. = 0.07–0.18, Table 2). Allele frequencies and pairwise F_{ST} values differed significantly between all pairs of sampling sites except between the closest sites, Anjalankoski E and W (Table 3).

There was significant association between geographical distance and pairwise F_{ST} matrices (Fig. 2, simple Mantel test: $r = 0.69$, $p = 0.002$). Pairwise F_{ST} comparisons with Luoto, the isolated site on the peninsula, differed from other comparisons (Fig. 2, partial Mantel test: $r = 0.7$, $p = 0.0007$). The effect of isolation by geographical distance was significant after omitting Luoto from the data (simple Mantel test: $r = 0.8$, $p = 0.01$, between distance and F_{ST} matrices). Overall F_{ST} (0.10) was also significantly different from zero after omitting Luoto (99% c.l. = 0.06–0.14).

Table 2. *F*-statistics for each locus as a mean of eight flying squirrel sampling sites. Deviations from zero NS, $p > 0.05$, * $p < 0.05$, based on jackknifing over populations.

Locus	F_{IS}	F_{ST}
10	0.002[NS]	0.102*
41	−0.043[NS]	0.142*
74	−0.050*	0.034*
E10	0.028[NS]	0.079*
E5	−0.067[NS]	0.234*
E6	−0.042*	0.113*
E1	−0.008[NS]	0.059*
All	−0.023*	0.115*

Table 3. Differentiation between sampling sites. Upper diagonal: F_{ST} values between sampling site pairs and significance levels based on bootstrapping values (* $p < 0.05$, ** $p < 0.01$). Lower diagonal: results for exact probability tests after Fisher's combined probability over each locus for allele frequency differences among sampling site pairs (NS, $p > 0.05$, *** $p < 0.0001$).

	I	K	N	AE	AW	AI	M	L
I		0.064**	0.061**	0.083**	0.128**	0.150**	0.159**	0.270**
K	***		0.048**	0.048**	0.058*	0.116**	0.108*	0.196**
N	***	***		0.063**	0.085**	0.145**	0.122**	0.221**
AE	***	***	***		0.002[NS]	0.097**	0.130**	0.192**
AW	***	***	***	NS		0.166**	0.173**	0.261**
AI	***	***	***	***	***		0.125**	0.225**
M	***	***	***	***	***	***		0.153**
L	***	***	***	***	***	***	***	

Discussion

We found substantial genetic differentiation between flying squirrel sampling sites that was affected by isolation due to increasing geographical distance. Heterozygosity levels were similar in the flying squirrel as in other Sciurid species (Columbian ground squirrel (*Spermophilus columbianus*), Stevens *et al*. 1997, Alpine marmot (*Marmota marmota*), Goossens *et al*. 2001, Yellow-pine chipmunk (*Tamias amoenus*), Schulte-Hostedde *et al*. 2001, European red squirrel (*Sciurus vulgaris*), Trizio *et al*. 2005) and we found a slightly negative F_{IS} value over all loci. However, the latter result was significant only for one site (with largest *n*) and the interpretation of this result is complicated. Several explanations are possible for the observed heterozygote excess in the flying squirrel: avoidance of inbreeding (Schwartz & Armitage 1980), polygynous mating system (Storz *et al*. 2001), small population size and bottlenecks (Cornuet & Luikart 1996, Pudovkin *et al*. 1996), genetic fluctuations in a metapopulation (Stewart *et al*. 1999), and sex biased dispersal (Prout 1981).

In small mammals, observations of genetic differentiation between populations are not uncommon even at short distances in microsatellite studies (between 2–20 km: house mice (*Mus musculus*), Stewart *et al*. 1999, Piute ground squirrel (*Spermophilus mollis*), Antolin *et al*. 2001, yellow-pine chipmunks (*Tamias amoenus*), Schulte-Hostedde *et al*. 2001). However, differentiation in the flying squirrel was relatively high as compared with, for example, that in another squirrel species occupying boreal forests, the red squirrel (*Sciurus vulgaris*) (Trizio *et al*. 2005, overall F_{ST} 0.04 for sites separated by 1.1–276 km in Italy). A similar result was found based on mtDNA: Uimaniemi (2004) compared mtDNA of red squirrels and flying squirrels in Finland and found lower genetic variation in the flying squirrel as well as high differentiation between flying squirrel sampling sites.

Genetic differentiation in the flying squirrel was also substantial at short distances, as compared with species dispersal distances. Between the closest sampling sites, Anjalankoski W and E, dispersal should be frequent, and this was
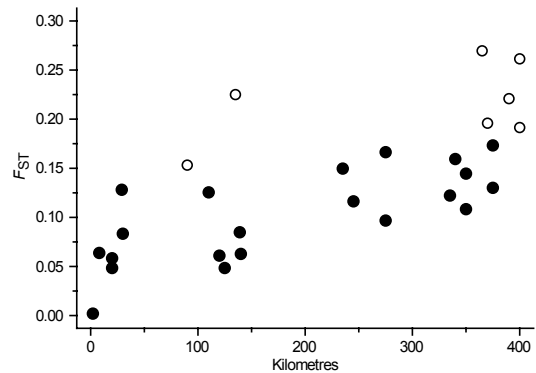
Fig. 2. Isolation by distance in pairwise F_{ST} comparisons for flying squirrel sampling sites. Open circles are comparisons with Luoto (the isolated site on the peninsula).

confirmed, as there was no genetic differentiation between these sites. However, the differentiation was already substantial between the second closest sampling sites, Iitti and Kuusankoski, separated by 8 km. This distance falls within the maximum dispersal distance of juvenile flying squirrels (Selonen & Hanski 2004). There are fields and one large lake between these sites, which may partly explain the differentiation. However, occasional gene flow between the sites should be possible.

Isolation by distance amongst our sampling sites fits the assumption that more geographically distant populations should be more differentiated than less geographically distant ones (Slatkin 1987). This has also been observed in other small mammal studies (Goossens *et al*. 2001, Trizio *et al*. 2005). Opposite results also occur, as in the white-footed mouse (*Peromuscys leucopus*), for example, there was no evidence of isolation (Mossman & Waser 2001) at a large geographic scale. As isolation by distance is associated with species movement abilities, in species that are able to move over large distances this effect is much weaker (e.g. noctule bat (*Nyctalus noctula*), Petit & Mayer 1999). Barriers for gene flow may also result in a lack of correlation between geographical distance and genetic differentiation (Neumann *et al*. 2004, Trizio *et al*. 2005). In the common hamster (*Cricetus cricetus*), the lack of isolation by distance was explained by the presence of barriers for gene flow (Neumann *et al*. 2004).

In our study, barriers for gene flow increased differentiation and resulted in low allele richness for the Luoto study site, the population on the peninsula in the Gulf of Bothnia. This is related to the geographic isolation of the area from the mainland and/or the recent colonization of the area (due to land uplift this peninsula has probably been an island a few hundred years ago). Whether this genetic isolation has some effects for survival of flying squirrels in Luoto remains unknown. Decreased genetic variation might indicate increased probability of extinction (Saccheri *et al*. 1998), although this is not necessarily the case (Groombridge *et al*. 2000).

On the mainland, most of the Finnish flying squirrel population should be structurally connected, because Finland is still mostly covered by forest — 68% of the land area is forest, 10% lakes and 6% fields (Finnish Forest Research Institute, 2000). Small-scale landscape patterns, such as fields, watercourses and urban areas, probably have some effect on the genetic structuring of the flying squirrel population, as found for other species (Gerlach & Musolf 2000). However, there are no such barriers that would totally isolate some parts of the southern Finnish flying squirrel population from other parts. Thus, despite good dispersal abilities and the apparent lack of structural isolation between most of the populations (Selonen & Hanski 2004), the fairly large differentiation between sampling sites in the flying squirrels indicates that gene flow over large distances is low (*see* also Uimaniemi 2004).

High differentiation between sites is probably associated with the decreased gene flow due to a small number of good quality spruce forest patches for dispersers to settle in. Consequently many dispersers settle in patches where the habitat quality is low, i.e., there are no cavities and the number of deciduous trees is low (Selonen & Hanski 2004). It is doubtful whether long-term survival, and thus occupancy, of these patches is possible. These patches of poor quality may function as habitat sinks that prevent dispersal between good quality patches and reduce the gene flow over large areas. For the conservation of flying squirrels this indicates that managers should ensure that there are enough good quality habitats in the landscape to enable gene flow over large areas. Thus our results, combined with those of Reunanen *et al*. (2000) and Selonen & Hanski (2003, 2004), emphasize the importance of landscape-level management in the conservation of the flying squirrel.

Acknowledgements

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