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Genetic differentiation among populations of the beetle *Bolitophagus reticulatus* (Coleoptera: Tenebrionidae) in a fragmented and a continuous landscape

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The effect of habitat fragmentation on genetic differentiation among local populations of the fungivorous beetle *Bolitophagus reticulatus* (Coleoptera: Tenebrionidae) was studied in two contrasting landscapes: one heavily fragmented with forest fragments of variable size surrounded by inhabitable agricultural fields, the other an old forest providing a continuous habitat. The genetic structure of the beetle within each of the two contrasting areas was investigated by means of protein electrophoresis, screening four polymorphic loci in 20 populations from each area. In both areas there were significant genetic differences among local populations, but on average differentiation in the fragmented area was three times greater than in the continuous one, strongly indicating a genetic isolation effect of habitat fragmentation. These genetic results are in accordance with previous studies on dispersal in this species.

Keywords: *Bolitophagus reticulatus*, dispersal, electrophoresis, fragmentation, genetic differentiation.

Introduction

In Europe, forest fragmentation has progressed especially far where agriculture dominates (Jennersten *et al.*, 1997), and predicting the effects of such fragmentation and isolation on population viability is currently one of the main challenges to landscape ecology and conservation biology (Meffe & Carroll, 1997). Metapopulation theory has been used widely to derive such predictions (Hanski & Simberloff, 1997). A key parameter in a metapopulation is the migration (or, more properly, dispersal) rate. Sufficient dispersal among habitat patches is a necessary condition for metapopulation persistence (Levins, 1969, 1970). Colonization or recolonization is necessary to compensate for local extinctions (Levins, 1969, 1970; Ås *et al.*, 1992; Hanski *et al.*, 1995), but dispersal may also provide rescue effects for extant extinction-prone populations (Brown & Kodrick-Brown, 1977). Dispersal and gene flow among populations are also of importance for maintenance of local genetic variability against loss caused by random genetic drift. Genetic variation is important because it enables the populations to adapt to changing environmental conditions (e.g. Meffe & Carroll, 1997). Also, loss of genetic variation in isolated populations is closely associated with increased inbreeding, which may introduce inbreeding depression and reduce population viability (Frankham, 1995; Saccheri *et al.*, 1998).

The number of dispersers among habitat patches is expected to decrease with increasing patch isolation because of the dilution effect associated with the spread of individuals in space (Ims, 1995) and mortality of individuals in the migration phase (Hanski *et al.*, 1994). Empirical studies may demonstrate effects of habitat isolation on dispersal in three ways. The first and most commonly employed approach analyses incidence rates (i.e. frequency of occurrence) in relation to distance (Rukke & Midtgaard, 1998; Kehler & Bondrup-Nielsen, 1999). The second alternative is to study dispersal directly by marking and recapturing individuals (e.g. Nilsson, 1997a). This latter approach will probably yield the least biased estimates of contemporary dispersal rates but usually requires efforts beyond the logistic and technical capacities of many field studies (Ims & Yoccoz, 1997). The third approach demonstrates the effects of isolation by distance from analyses of the genetic structure among populations in habitat patches. This genetic approach also gives information about potential loss of genetic variation resulting from fragmentation. As the three approaches for studying dispersal have

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their own difficulties and potential pitfalls, a combined approach may be useful (Ims & Yoccoz, 1997). Species that are numerous and widespread are most amenable to such combined approaches (Ås *et al.*, 1992). Generalizations to rarer, and presumably more vulnerable, species can be made through careful extrapolation (Wiens *et al.*, 1993). The rationale behind this is that if negative effects of fragmentation are apparent in a common species, then the picture is probably even more severe for more scarce species.

The main objective of the present study is to check whether human-induced landscape fragmentation has affected the genetic population structure of the beetle *Bolitophagus reticulatus* (Coleoptera: Tenebrionidae). We apply enzyme electrophoresis to characterize and compare genetic differentiation patterns within and between local populations of the beetle in two contrasting landscapes; one is a heavily fragmented agricultural landscape and the other a continuous forest. The effects of habitat fragmentation found in this genetic survey are compared to the results obtained from previous analyses of incidence rates in the species (Rukke & Midtgaard, 1998; Sverdrup-Thygeson & Midtgaard, 1998).

Materials and methods

Study areas

This study was conducted in two contrasting areas situated approximately 110 km apart (Fig. 1). The first area, in Lierdalen (59°48′N, 10°16′E), is in an old agricultural landscape that has been cultivated for approximately 4500 years and extensively so during the last 1500–2000 years (Løvik & Puschmann, 1989). In this area, forest fragments mainly of deciduous trees lie scattered like islands of variable sizes within a matrix consisting of cultivated fields (Fig. 1a). The forest fragments have been left more or less undisturbed because they occur in gullies where the ground is unsuitable for agriculture. The other area, at Noresund (60°11′N, 9°38′E), lies in an old, continuous forest with lots of decaying wood and few human traces. A steep forest hillside unsuitable for commercial logging constitutes the study area (Fig. 1b), although we cannot exclude the possibility of some small-scale logging in the past. The forest in Noresund is a mixture of deciduous (mostly birch) and coniferous trees. The climate in both areas is cold temperate according to Köppen's climate zones (Wallén, 1970).

The study species

Bolitophagus reticulatus is a monophagous fungivore beetle inhabiting the fruiting bodies of the tinder fungus (*Fomes fomentarius*) (Hansen, 1945; Benick, 1952). Mainly dead basidiocarps (*sensu* Matthewman & Pielou, 1971) are exploited by the beetle (Midtgaard *et al.*, 1998), but adults are also frequently observed under living basidiocarps, probably feeding on spores (Nilsson, 1997b). Basidiocarps may persist for several years after their death (Gilbertson, 1984) and can function as a habitat for the beetle during this time. A single tree can provide dead basidiocarps for years and, hence, function as a reasonably stable habitat for the beetle over several generations. Because the larvae, pupae and adults are found together within the basidiocarps in the spring, it has been proposed that the beetle has a two-year life-cycle (Hansen, 1945). Females lay 24–28 eggs on top of the basidiocarp, and individuals live up to 4 years (Nilsson, 1997b). Results from capture–recapture and incidence studies show that dispersal occurs primarily between basidiocarps within the same tree (Nilsson, 1997b), and that the occurrence of *B. reticulatus* in trees decreases significantly with distance to the nearest inhabited trees (Rukke & Midtgaard, 1998).

Sampling design

Collection of beetles was undertaken from April to August 1997. Individuals living within the basidiocarps of a single tree were presumed to represent a single local population of *B. reticulatus* (Nilsson, 1997b; Rukke & Midtgaard, 1998), and the sampling protocol was designed accordingly.

In Lierdalen all the forest fragments in the study area were examined thoroughly for presence of basidiocarps. All dead basidiocarps were collected and trees harbouring basidiocarps with a total number of at least 25 larvae were included in the genetic analyses. The limit of 25 larvae was set to get a reasonable number of individuals from each population for statistical analyses. Twenty populations (i.e. trees) with 25 or more larvae were found, distributed in 11 of the 19 forest fragments in the study area (totalling 1009 individuals; about 500 from each study area). The number of sampled populations varied from one to three in the 11 fragments (cf. Fig. 1a). At Noresund, which has a much higher incidence of beetles (pers. obs.), the same total number of populations (20) and larvae per population (about 25) as in Lierdalen was collected. The geographical scales of the two sample areas are comparable (cf. Fig. 1a,b), although a somewhat greater range was sampled in Lierdalen on account of the lower density of beetles there. The maximum distance between populations in Lierdalen was 4.4 km, as compared to 1.84 km in Noresund [average distances between populations were 1958 m (SD 992 m) and 635 m (SD 405 m), respectively].

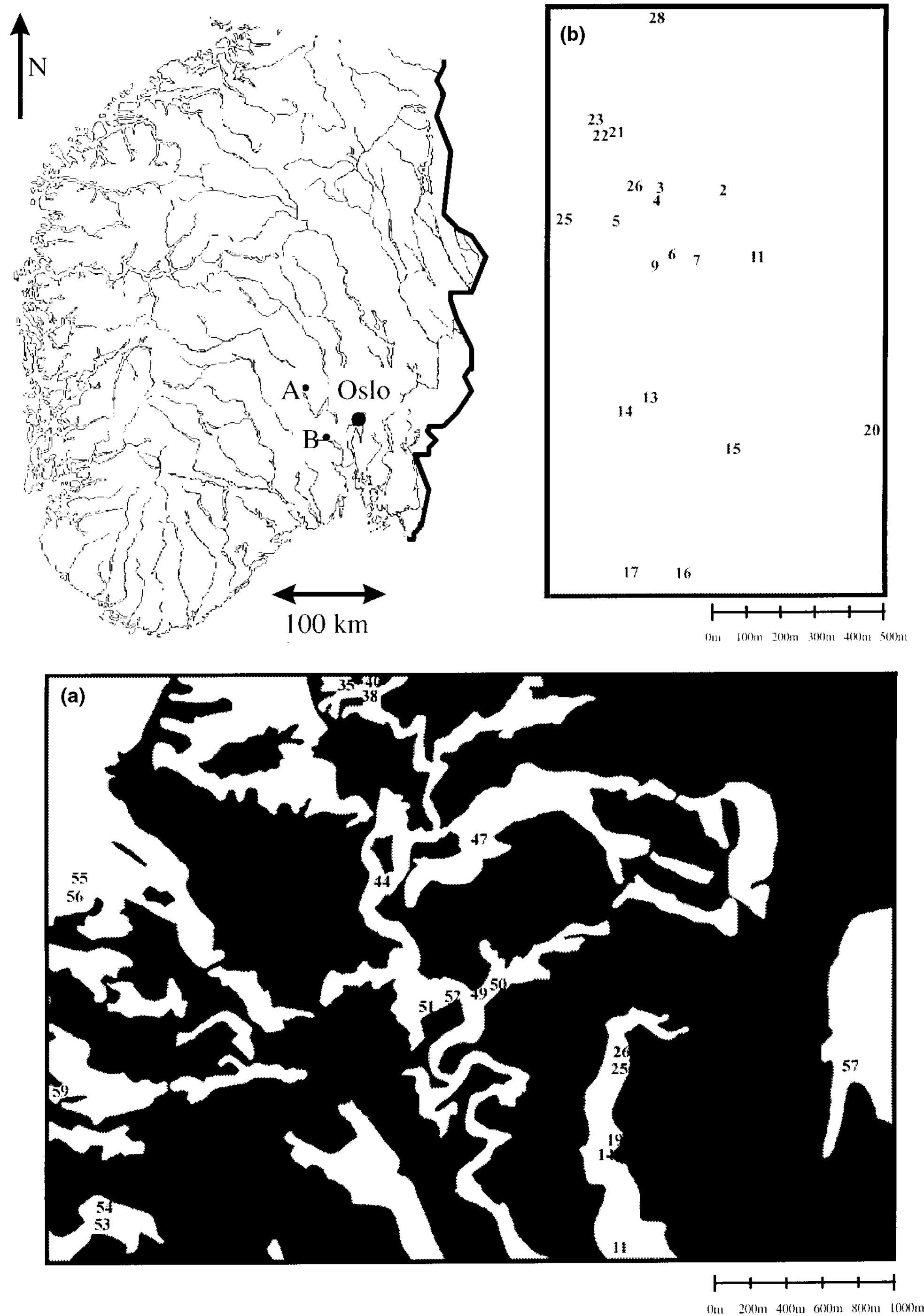

Fig. 1 Map showing the location of the two study-areas in southern Norway (black dots), with detailed site maps of the fragmented area (a) Lierdalen, and the continuous study area, (b) Noresund. White background represents forest, whereas black is inhabitable areas, consisting of mainly agricultural fields. The numbers identify separate trees (cf. Appendices). Note the different scales of maps (a) and (b).

After collection, all basidiocarps were preserved in a cold-storage chamber at 10°C until larvae were removed, within a week. Living larvae were frozen separately and kept at −80°C prior to electrophoretic analyses.

Electrophoretic analyses

Horizontal starch gel electrophoresis was used to assess protein polymorphism. Electrophoresis and staining

procedures followed conventional protocols (e.g. Hillis & Moritz, 1990). Of 29 enzymes screened, 10 enzymes, presumably coded for by 10 gene loci, were found to have satisfactory resolution and activity for routine screening of genetic variability. Of these 10 loci four turned out to be polymorphic (*ACON*, *PGM*, *EST-1* and *GPI*) and were used in the subsequent analyses. The enzymes ACON (EC 4.2.1.3) and PGM (EC 5.4.2.2) were run on a tris-borate buffer at pH 8.3 (modified from Selander & Yang, 1969), whereas EST (EC 3.1.1.*) and GPI (EC 5.3.1.9) were stained on gels made with a tris-phosphate buffer at pH 8.0 (modified from Guyomard & Krieg, 1983). Genotypes were inferred from the banding patterns on the basis of conformity with known protein structure (Hillis & Moritz, 1990).

Statistical analysis

Allele frequencies within populations were estimated from genotypes by gene counting. Heterogeneity of allele frequencies among populations within each area was tested against the null hypothesis of equal allele frequencies in all sampled populations (trees) with the contingency chi-squared test, pooling rare alleles where appropriate. Overall amounts of genetic variability within populations and areas were characterized using average heterozygosities ('gene diversity', H_S and H_T: Nei & Chesser, 1983).

For analyses of genetic structure within and among areas Wright's *F*-statistics were estimated according to Weir & Cockerham (1984: eqns 1–4 and 10). In order to check for correlation in gene frequency with geographical distance, F_{ST} was also estimated separately for all pairs of populations within each area. A positive regression of pairwise F_{ST} with distance indicates an 'isolation-by-distance' effect (Rousset, 1997), and this was tested for using the permutation procedure in the GENEPOP software 3.1a package (Raymond & Rousset, 1995). The pairwise F_{ST}-estimates were also used to compare the amount of differentiation in the two areas by averaging over pairs at comparable geographical distances, taking care of the somewhat different sample scales in the two areas (above). This was achieved by calculating an average F_{ST} over pairwise values using only population pairs situated at or below 1840 m in the fragmented area when comparing F_{ST} with the continuous one, which includes pairs up to this distance. We used a randomization test to compare these two averages; randomly resampling pairwise F_{ST} with replacement 10 000 times for each area.

Results

The same four gene loci were polymorphic in both the continuous (Noresund) and the fragmented (Lierdalen) areas. With the exception of a few rare alleles, the same alleles were segregating in both areas (cf. Appendices A and B). Genotype proportions within populations (that is, single trees) generally conformed to Hardy–Weinberg expectations, with the sole exception of *EST-1* in population 49 in Lierdalen, which displayed a slightly significant excess of heterozygotes. The general conformity with Hardy–Weinberg expectations is reflected in average F_{IS} estimates being close to zero for all loci in both areas (Table 1); this is in accordance with the notion that beetles inhabiting basidiocarps on the same tree represent the same biological population.

The total beetle populations in the two areas are comparable genetically. First, the total amount of genetic variation, as measured by the average heterozygosity (H_T), is quite similar in the two areas (0.248 vs. 0.207; Table 1). Secondly, the average F_{ST} between the two areas, pooling all populations within each area, is very low ($F_{ST} = 0.0047$) although statistically significant ($\chi^2_5 = 53.4$, $P \ll 0.001$; combined allele frequency heterogeneity test over all loci).

There is a significant difference between areas in the degree of genetic differentiation among local populations (trees). In both areas allele frequencies among populations differ significantly (see Appendices), but the differences are considerably greater in the fragmented area at all four loci. This is reflected in the larger average F_{ST} among populations in the fragmented area (0.069 as compared to 0.023 in the continuous area) and holds also for each locus considered separately (Table 1).

The regression of pairwise F_{ST}-values against distance was very low in each area with $b = -0.14$ and 0.084 in the continuous and the fragmented area, respectively; neither was significantly different from zero (Mantel tests: $P > 0.1$ for each area). This apparent lack of a relationship between genetic differentiation and distance indicates that the somewhat different geographical scales (above) cannot explain the observed difference in average F_{ST} within the two areas. This conclusion was further supported by the average pairwise F_{ST}-values based on pairs situated below 1840 m (Table 2). These average F_{ST}s are nearly identical to those calculated previously over all populations and distances (cf. Tables 1 and 2), demonstrating that populations in the fragmented area are indeed more differentiated than in the continuous one even after correcting for geographical scale. Figure 2 depicts the distributions for each area of 10 000 averages of randomly resampled pairwise F_{ST}-values at the same geographical scale. The complete absence of any overlap between the two distributions shows that the difference in F_{ST} for the areas is highly significant, and that different sampling scales cannot explain the observed difference in F_{ST} for the two areas.

Table 1 Estimates of genetic variability (H_T, H_S) and fixation indices (F_{IS}, F_{IT}, F_{ST}) in *Bolitophagus reticulatus* within and among populations (trees) in the two areas

		Genetic variability†		F-statistics‡		
Area	Locus	H_T	H_S	F_{IS}	F_{IT}	F_{ST}
Lierdalen (fragmented)	*ACON*	0.265	0.244	−0.073	−0.007	0.061
	PGM	0.187	0.170	−0.028	0.051	0.077
	EST	0.476	0.434	−0.013	0.060	0.073
	GPI	0.063	0.059	0.044	0.096	0.055
	Mean	0.248	0.227	−0.028	0.043	0.069
	SD	0.173	0.158	0.048	0.037	0.009
Noresund (continuous)	*ACON*	0.254	0.241	−0.010	0.022	0.032
	PGM	0.079	0.075	−0.081	−0.039	0.039
	EST	0.481	0.465	0.033	0.048	0.016
	GPI	0.014	0.014	−0.018	−0.005	0.013
	Mean	0.207	0.199	0.008	0.031	0.023
	SD	0.209	0.202	0.047	0.037	0.013

†Estimated according to Nei & Chesser (1983).
‡Estimated according to Weir & Cockerham (1984) ($F_{IS} = f$, $F_{IT} = F$, $F_{ST} = \theta$).

Table 2 Comparison of genetic differentiation (F_{ST}, averaged over pairs of populations) for the continuous (Noresund) and fragmented (Lierdalen) area. Differences in average pairwise F_{ST} between the two areas were tested by means of resampling single-pair F_{ST} values. For Lierdalen this was carried out twice: using all population pairs and using only pairs situated below 1840 m to match the distances in Noresund

Area	Population pairs	Average pairwise F_{ST}	Noresund vs. Lierdalen
Noresund	190	0.023	—
Lierdalen (all pairs)	190	0.069	$P \ll 0.001$
Lierdalen (distance < 1840 m)	102	0.067	$P \ll 0.001$

Discussion

The present study is among the first to investigate genetic differentiation contrasting a large continuous landscape with a fragmented area (but see van Dongen *et al*., 1998). We found that local *B. reticulatus* populations, inhabiting different trees, were genetically differentiated in both areas. However, the level of genetic differentiation among populations, as estimated by F_{ST}, was considerably greater in the fragmented agricultural area than in the continuous forest. The finding that all loci displayed larger genetic differentiation in the fragmented area indicates that a common mechanism is acting equally on all loci; this is most likely random genetic drift rather than, say, natural selection which should act differently on each locus. Hence, the observed elevated genetic differentiation is probably a direct consequence of genetic drift in conjunction with reduced dispersal following habitat fragmentation.

From estimated incidence rates in the fragmented area, which partly overlaps our study area in Lierdalen, Rukke & Midtgaard (1998) found that the fragmented area possessed a lower incidence of *B. reticulatus* among trees than the continuous area studied by Sverdrup-Thygeson & Midtgaard (1998). These results are in general agreement with those of Nilsson (1997a), who found that the probability of *B. reticulatus* dispersing between trees with basidiocarps increased with density of trees, which is, of course, higher in continuous areas.

It is difficult to identify whether landscape fragmentation increases genetic differentiation because of a direct isolation effect, i.e. that the agricultural fields present barriers to movement, or more indirectly through the thinning of inhabited trees, i.e. an 'isolation-by-distance' effect. We did not find any statistically significant trend in genetic differentiation with distance in the present study and, hence, there is no clear evidence for isolation by distance either in the fragmented or in the continuous area. This does not necessarily mean that there is no such effect, however, because there may have been insufficient time for the expected genetic pattern to build up. This is especially so

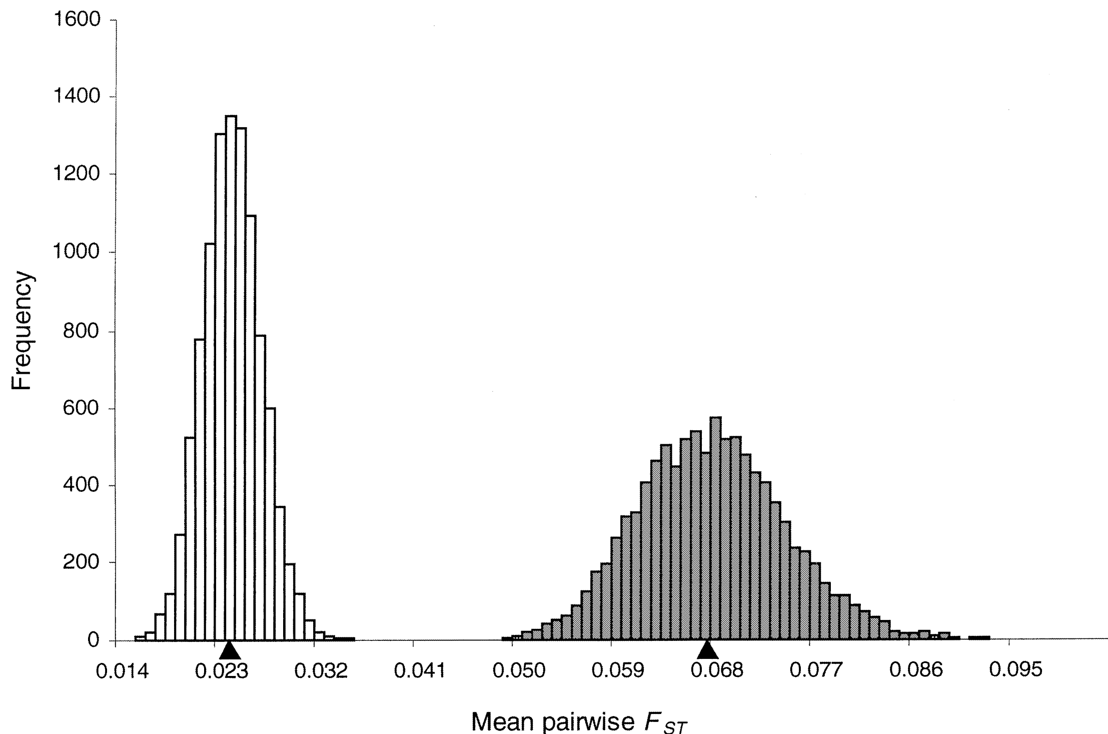

Fig. 2 Distribution of 10 000 averages of randomly resampled pairwise F_{ST}-values from Noresund (open bars) and Lierdalen (filled bars), using the same geographical scale for both areas (below 1840 m). Black triangles indicate mean F_{ST} for each area.

if occasional fires, logging or other potential large-scale habitat destruction have occurred in the recent past, temporarily pushing the system away from equilibrium. On the other hand, Rukke & Midtgaard (1998) found that the frequency of inhabited trees reduced with increasing distances to neighbouring inhabited trees up to a distance of only about 30 m. Beyond that they found no apparent relationship between distance and dispersal. Because only a few of the populations in the present study were closer than 30 m, we would expect little or no trend of genetic differentiation with distance. Hence, the expected — as well as the observed — pattern of genetic differentiation appears to conform more closely to an 'island' model of dispersal than to one of 'isolation-by-distance'. If so, the greater level of genetic differentiation in the fragmented area should arise largely as a consequence of reduced numbers of (successful) dispersers, rather than by limiting the distance that they travel. Interestingly, van Dongen *et al.* (1998), studying genetic differentiation in another insect species, also failed to detect an isolation-by-distance effect in either fragmented or continuous areas.

The colonization of newly basidiocarp-inhabited trees highlights a different aspect of dispersal. Because the basidiocarp eventually decays and fails to provide a habitat, the beetle is periodically forced to colonize new trees. The resultant abandoning (or extinction) vs. colonization dynamics of the *B. reticulatus–F. fomentarius* system is expected to enhance gene flow and, consequently, to break down genetic differentiation. However, the nature of the colonization events is dual: a vacant patch is likely to be colonized by far fewer individuals than it can support (McCauley, 1995), making founder effects an important contributor to increased genetic differentiation. McCauley (1995) concluded in his review on metapopulations genetics that when there is a frequent turnover of local populations, the distribution of genetic variation within and among fragments would be determined largely by the colonization process and less by postcolonization events (e.g. accumulated genetic drift). Our results may be in agreement with these considerations because the predicted reduced number of dispersers (or colonizers) in the fragmented area should result in a higher level of genetic differentiation in that area, as observed.

This study has demonstrated that in *B. reticulatus*, an abundant and widespread species in the boreal forest, the genetic differentiation among local populations was higher in a fragmented than in a continuous forest landscape. Although our results are in qualitative agreement with population genetics theory, few empirical studies have thus far compared genetic differentiation in populations living under nearly pristine conditions with those living in a heavily fragmented habitat. Such

studies are important because real populations are infinitely more complex than theoretical ones, and there is the need to check the relevance of particular theoretical models for natural populations. Furthermore, empirical studies of common species, as the beetle considered here, may serve as model organisms for other, perhaps rare or endangered, species where similar comparison would be impossible. Thus, common species may have an important role in conservation biology in bridging the gap between theory and field study of conservation targets. This study has shown that human-induced forest fragmentation may incur consequences for the genetic structure of forest insects, and that habitat fragmentation represents a potential threat for species with restricted dispersal abilities. Although the observed elevated genetic differentiation for the screened genes is in itself of limited consequence, the genome-wide implications are reduced local (and global) effective population sizes, and loss of alleles that could be advantageous and fixation of disadvantageous ones. The reduced demographic and genetic connectivity following fragmentation could therefore ultimately increase the chances of extinction.

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Appendix A

Allele frequencies at four isozyme loci in 20 beetle populations in the fragmented area in Lierdalen (cf. Fig. 1a), and χ^2-test of allele frequency heterogeneity (*** $P < 0.001$)

Tree number	Coordinates		Sample size	*ACON*			*PGM*			*EST*				*GPI*			
	X	*Y*		100	80	110	100	80	120	100	80	110	60	100	80	120	110
11	1202	374	25	0.94	0.06	0	0.82	0.18	0	0.56	0.44	0	0	0.94	0.04	0.02	0
14	1320	977	25	1.00	0	0	0.92	0.08	0	0.28	0.72	0	0	1.00	0	0	0
19	1328	1003	25	0.84	0.14	0.02	0.92	0.08	0	0.46	0.54	0	0	1.00	0	0	0
25	1314	1515	25	0.64	0.36	0	0.86	0.14	0	0.72	0.28	0	0	1.00	0	0	0
26	1277	1546	30	0.87	0.13	0	0.88	0.12	0	0.58	0.42	0	0	1.00	0	0	0
35	3377	3482	25	0.98	0.02	0	0.90	0.10	0	0.64	0.36	0	0	0.90	0.10	0	0
38	3228	3432	25	0.83	0.17	0	0.98	0.02	0	0.56	0.44	0	0	0.94	0.06	0	0
40	3125	3427	25	0.88	0.12	0	0.94	0.06	0	0.90	0.10	0	0	0.94	0.06	0	0
44	3106	2418	25	0.94	0.06	0	0.92	0.08	0	0.58	0.42	0	0	0.90	0.10	0	0
47	2447	2612	25	0.88	0.10	0.02	0.82	0.18	0	0.62	0.38	0	0	0.98	0.02	0	0
49	2606	1800	25	0.90	0.10	0	0.78	0.22	0	0.54	0.46	0	0	0.96	0.04	0	0
50	2511	1792	25	0.76	0.24	0	0.80	0.20	0	0.86	0.14	0	0	1.00	0	0	0
51	2933	1650	25	0.62	0.38	0	0.68	0.32	0	0.60	0.40	0	0	1.00	0	0	0
52	2702	1776	30	0.83	0.17	0	0.75	0.25	0	0.43	0.57	0	0	0.97	0.03	0	0
53	3992	648	25	0.76	0.24	0	1.00	0	0	0.72	0.28	0	0	1.00	0	0	0
54	3969	667	25	0.78	0.22	0	0.94	0.06	0	0.56	0.44	0	0	1.00	0	0	0
55	4242	2381	25	0.78	0.20	0.02	1.00	0	0	0.58	0.42	0	0	1.00	0	0	0
56	4267	2306	25	0.82	0.18	0	1.00	0	0	0.72	0.28	0	0	0.82	0.18	0	0
57	38	1428	25	0.98	0.02	0	1.00	0	0	0.80	0.20	0	0	1.00	0	0	0
59	4440	1286	25	0.84	0.16	0	1.00	0	0	0.48	0.52	0	0	1.00	0	0	0
Test for heterogeneity				$\chi^2 = 99.1$***			$\chi^2 = 93.3$***			$\chi^2 = 94.2$***				$\chi^2 = 97.4$***			

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Appendix B

Allele frequencies at four isozyme loci in 20 beetle populations in the continuous area at Noresund (Fig. 1b), and χ^2-test of allele frequency heterogeneity (*$P < 0.05$, ***$P < 0.001$)

Tree number	Coordinates		Sample size	*ACON*			*PGM*			*EST*				*GPI*			
	X	*Y*		100	80	110	100	80	120	100	80	110	60	100	80	120	110
2	696	1674	25	0.74	0.26	0	1.00	0	0	0.78	0.22	0	0	1.00	0	0	0
3	525	1674	25	0.88	0.12	0	0.94	0.06	0	0.52	0.48	0	0	1.00	0	0	0
4	518	1661	25	0.66	0.28	0.06	1.00	0	0	0.54	0.46	0	0	0.94	0.06	0	0
5	401	1568	25	0.86	0.14	0	0.96	0.04	0	0.56	0.34	0	0.10	1.00	0	0	0
6	564	1458	25	0.86	0.14	0	0.98	0.02	0	0.54	0.46	0	0	1.00	0	0	0
7	623	1447	25	1.00	0	0	1.00	0	0	0.78	0.22	0	0	1.00	0	0	0
9	511	1423	25	0.78	0.22	0	0.96	0.04	0	0.60	0.40	0	0	1.00	0	0	0
11	785	1461	25	0.78	0.22	0	0.94	0.06	0	0.56	0.44	0	0	0.98	0	0	0.02
13	483	988	25	0.80	0.16	0.04	0.92	0.08	0	0.68	0.32	0	0	0.98	0.02	0	0
14	413	945	25	0.76	0.22	0.02	0.98	0.02	0	0.68	0.32	0	0	1.00	0	0	0
15	714	819	25	0.92	0.08	0	0.98	0.02	0	0.42	0.56	0.02	0	1.00	0	0	0
16	578	411	25	0.82	0.16	0.02	0.92	0.08	0	0.62	0.38	0	0	1.00	0	0	0
17	435	415	25	0.96	0.04	0	0.96	0.02	0.02	0.56	0.44	0	0	1.00	0	0	0
20	1102	889	25	0.90	0.10	0	0.96	0.04	0	0.68	0.32	0	0	0.98	0.02	0	0
21	371	1866	24	0.94	0.06	0	1.00	0	0	0.69	0.31	0	0	1.00	0	0	0
22	364	1864	25	0.90	0.08	0.02	1.00	0	0	0.64	0.36	0	0	1.00	0	0	0
23	348	1895	25	0.80	0.18	0.02	1.00	0	0	0.56	0.44	0	0	1.00	0	0	0
25	241	1575	25	0.86	0.14	0	0.92	0.08	0	0.52	0.48	0	0	1.00	0	0	0
26	442	1697	25	0.93	0.07	0	0.80	0.20	0	0.64	0.34	0	0.02	0.98	0.02	0	0
28	505	2248	25	0.90	0.10	0	0.96	0.04	0	0.70	0.26	0.04	0	1.00	0	0	0
Test for heterogeneity				$\chi^2 = 144.8$***			$\chi^2 = 123.0$***			$\chi^2 = 145.2$***				$\chi^2 = 54.2$*			