

1 **Recent habitat fragmentation due to roads can lead to significant**  
2 **genetic differentiation in an abundant flightless ground beetle**

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21 Running title: Differentiation due to habitat fragmentation

1 **Abstract**

2 Although habitat fragmentation is suspected to pose a major threat to biodiversity, its  
3 impact on abundant invertebrate species has been little investigated. We assessed the  
4 genetic population structure of the flightless ground beetle *Abax parallelepipedus* in a  
5 forest fragmented by two main roads and a highway using five microsatellite loci. We  
6 detected low levels of genetic differentiation, which was concordant with the high  
7 population densities of 632 - 1707 individuals/ha estimated with a mark-recapture  
8 method. A Mantel test detected a highly significant increase of pairwise  $F_{ST}$ -values  
9 with the number of roads between sampling locations. As expected, the most  
10 pronounced effect of the isolation due to roads was observed in the sample from the  
11 smallest fragment (highway exit loop), which was significantly differentiated from  
12 most other locations. However, no signs of a recent bottleneck or a loss of genetic  
13 variability were detected in this population, indicating a still relatively large effective  
14 population size ( $N_e$ ). Computer simulations confirmed that the observed  $F_{ST}$ -values  
15 were indeed compatible with a  $N_e$  of a few hundred individuals in this fragment,  
16 assuming strong or absolute isolation since the construction of the roads. We discuss  
17 the implications of our findings for the conservation of abundant but poorly dispersing  
18 species in fragmented habitats.

19

20

## 1 **Introduction**

2 The loss of natural habitats caused by human activities is one of the major threats to  
3 the long-term persistence of many species. The remaining habitat patches are often  
4 small and isolated from each other by less suitable landscape elements, such as  
5 agricultural areas, settlements and roads. Potentially negative effects of fragmentation  
6 include the simple reduction of habitat area, modifications of the physical  
7 environment and increased isolation of local populations (Primack 1993). The latter is  
8 of particular significance for animals or plants with limited dispersal capabilities and  
9 may increase the risk of extinction because of different demographic and genetic  
10 factors. For example, due to the destruction of effective metapopulation structures,  
11 stochastic fluctuations in the size of local populations cannot be buffered by  
12 immigrants (den Boer 1990). Relevant genetic factors comprise the accelerated loss of  
13 genetic variability due to random genetic drift and a potentially increased level of  
14 inbreeding in small remnant populations (Hartl & Clark 1997). The relative  
15 significance of these different aspects for the persistence of fragmented populations is  
16 unresolved (Lande 1988; Amos & Balmford 2001), but several studies showed that  
17 both demographic and genetic factors may be important (Gonzalez *et al.* 1998;  
18 Brooker & Brooker 2002; Keller & Waller 2002). As the strength of the isolation  
19 between local populations is central to all scenarios, its assessment will be essential  
20 irrespective of the exact mechanisms leading to extinction.

21 Ground beetles (Coleoptera, Carabidae) are useful organisms to evaluate the effects of  
22 fragmentation on insects, because their biology is well known, and the taxon  
23 comprises a large number of species with different dispersal abilities and habitat  
24 preferences (Thiele 1977). So far, several studies have shown that fragmentation may  
25 change the species composition in ground beetle communities, for example, by  
26 leading to the disappearance of habitat specialists (Halme & Niemelä 1993; Davies &  
27 Margules 1998; Abildsnes & Tommerås 2000; Niemelä 2001). Within species,  
28 population structure may be altered if the areas between fragments represent at least  
29 partial barriers to gene flow (Brouat *et al.* 2003). *Abax parallelepipedus* is a common  
30 forest species (Marggi 1992), but Assmann (1999) found that it was often absent from  
31 isolated or recent woodlots, possibly due to its flightlessness and consequently low  
32 powers of dispersal (Charrier *et al.* 1997). In fact, extensive field studies showed that  
33 even a relatively narrow artificial barrier in the form of a six metre wide road had a

1 very strong impact on the movement of *A. parallelepipedus* and was crossed by only  
2 one out of 742 recaptured individuals (Mader 1984). However, genetic analyses are  
3 needed to assess the extent of actual gene flow over longer time spans.

4 Recently, we showed for *Carabus violaceus*, a flightless ground beetle occurring at  
5 relatively low densities, that the isolation due to roads was indeed strong enough to  
6 lead to significant genetic differentiation within a relatively short period of time  
7 (Keller & Largiadèr 2003a). Such an increase of genetic differentiation at neutral loci  
8 in the absence of gene flow is a result of allele frequency changes due to random  
9 genetic drift. The intensity of genetic drift in a population is inversely related to its  
10 effective size ( $N_e$ ; Hedrick 2000), i.e. small populations will diverge faster than large  
11 ones. Because  $N_e$  may be substantially smaller than the census population size  
12 (Frankham 1995), it cannot be taken for granted that habitat fragmentation will only  
13 affect low-density species. In this study, we focused on the flightless ground beetle,  
14 *Abax parallelepipedus*, which was expected to reach considerably higher densities  
15 (1000-2000 individuals/ha; Loreau & Nolf 1993; Franceschini *et al.* 1997) than *C.*  
16 *violaceus* (88 individuals/ha; I. Keller, unpublished data).

17 For comparative reasons, the study was carried out in the same area as described in  
18 Keller & Largiadèr (2003a). The high population density of *A. parallelepipedus* was  
19 verified for this forest by means of a Jolly-Seber mark-recapture method (Jolly 1965).  
20 We then assessed whether the isolation due to roads was strong enough to lead to  
21 detectable divergence between populations on opposite sides by studying the genetic  
22 population structure based on microsatellite loci. We hypothesised that artificial  
23 barriers, i.e. roads, would have a stronger impact on genetic differentiation than  
24 geographical distance between study sites. Due to the inverse relationship between  
25 rate of divergence and effective population size, the barrier effect of roads should first  
26 be detectable in the smallest forest fragments. In addition to *F*-statistic based analyses,  
27 individual-based approaches were used to investigate the genotypic structure within  
28 the study area as these methods are expected to be more sensitive to recent changes in  
29 gene flow (e.g. Sunnucks 2000; Stow *et al.* 2001). We further tested for genetic  
30 evidence of a recent bottleneck and for a reduction of genetic variability in the  
31 presumably smallest populations. Finally, we used computer simulations to assess if  
32 the observed genetic differentiation between fragments was compatible with realistic  
33 population parameter values assuming that roads were strong barriers to gene flow.

1 **Materials and Methods**

2 *Study species*

3 *Abax parallelepipedus* (Piller & Mitterpacher, 1783; syn. *A. ater*) occurs in forests  
4 throughout northern and central Europe, and the polyphagous predator and scavenger  
5 is often the most abundant ground beetle in these habitats (Marggi 1992). The animals  
6 are 16-21 mm in length and do not have wings. Males can be distinguished from  
7 females on the basis of their broadened first tarsi (Wachmann *et al.* 1995). Although  
8 adults of *A. parallelepipedus* have been reported to live for more than one year  
9 (Loreau 1990), this appears to be rare in our study populations and an average  
10 generation time of one year is likely. The beetles are nocturnal (Thiele 1977) and  
11 active between February and October with an activity peak in July and August  
12 (Marggi 1992).

13

14 *Study area and tissue collection*

15 Our main study area was a mixed forest near Bern (46°57' N, 7°26' E; Switzerland),  
16 which was divided into differently sized fragments by a highway and two main roads  
17 (Fig. 1). The different forest patches were completely isolated from each other. The  
18 size of the fragments ranged between 1 ha and ca. 400 ha, and their age between 31  
19 and more than 130 years (see legend of Fig. 1 for details). The study sites were  
20 located in a part of the forest which was relatively flat and did not possess  
21 conspicuous topographic structures. Care was taken to ensure that the stretches of the  
22 roads directly between the study sites did not follow particular landscape elements  
23 such as for example brooks, which could represent much older, natural barriers to  
24 dispersing ground beetles. Therefore, the location of the roads is likely to be random  
25 with respect to other factors, which might play a role in determining the genetic  
26 structure of *A. parallelepipedus* populations. The cover of vegetation was quite  
27 homogenous throughout the study area.

28 To assess genetic population structure in a continuous habitat, we collected tissue  
29 samples from seven locations in fragment 2 (Fig. 1). This area may not be entirely  
30 representative of an unfragmented habitat as it is completely surrounded by roads,  
31 which might influence beetle movements and, consequently, genetic population  
32 structure. The advantage of using fragment 2 as a continuous control habitat, however,

1 is that it is characteristic of the study area with respect to factors such as topology,  
2 vegetation or management history.

3 Most of the Bernese samples included in the subsequent analyses were collected in  
4 2000, except for sample 7 (2001) and samples 2d, e, f, and g (2003). In a preliminary  
5 investigation, we assessed the temporal changes in allele frequencies at locations 2c  
6 and 4. At both site, two samples of 30 individuals were collected in two consecutive  
7 years. No significant differences in allele frequencies were detected between these  
8 temporal samples (site 2c:  $P = 0.89$ ; site 4:  $P = 0.54$ ; GENEPOP version 3.3). This  
9 indicates that the use of samples from different years is unlikely to have a strong  
10 impact on our analyses. The distance between two sampling sites varied between 200  
11 and 1750 m. As a reference for differentiation at a larger geographical scale, we  
12 included a sample from a location near Spiez (46°41' N, 7°39' E) at a distance of ca.  
13 40 km and a sample from Belgium (50°50' N, 4°20' E) at a distance of ca. 500 km  
14 from Bern.

15 At each sampling site, we installed 16 dry pitfall traps arranged in a grid of 4 x 4 at  
16 regular intervals of 5 m. The traps consisted of plastic cups with a diameter of 11.5  
17 cm and a depth of 14 cm and were emptied twice a week. For tissue collection, the  
18 captured beetles were taken to the lab, where they were anaesthetised with CO<sub>2</sub> before  
19 one middle tarsus was cut off. Nine treated and eight untreated individuals of *A.*  
20 *parallelepipedus* were kept in the laboratory for one month. During this period, only  
21 one untreated animal died, which indicated that the amputation of a tarsus did not  
22 increase mortality at least under laboratory conditions. This is likely to be similar in  
23 the field, as we regularly recaptured individuals with a missing tarsus. The collected  
24 tarsi were stored in absolute ethanol, and the beetles were released back into the forest  
25 on the same day.

26

### 27 *Microsatellite typing*

28 The beetle tarsi were ground with sterile plastic pestles, and the DNA was extracted  
29 with a Chelex resin extraction (Estoup *et al.* 1996). The number of individuals  
30 analysed per sampling site is indicated in Table 1. The sex ratio was balanced in all  
31 samples except 2f and 2g, which contained considerably more males than females.  
32 The sex of the Belgian animals was unknown. All animals were typed at the five

1 polymorphic microsatellite loci APA12499CMPG, APA05103CMPG,  
2 APA06272CMPG, APA14224CMPG, and APA14449CMPG (Keller & Largiadèr  
3 2003b). The genotyping was carried out using the PCR protocols and cycling  
4 conditions described in Keller & Largiadèr (2003b), and the PCR products were  
5 resolved on an automated DNA sequencer (ABI 3100).

6

### 7 *Genetic analyses*

8 We tested all samples for deviations from Hardy-Weinberg equilibrium using the  
9 approach of Guo & Thompson (1992) implemented in ARLEQUIN version 2.000  
10 (Schneider *et al.* 2000). The analyses were carried out with 1'000'000 steps in the  
11 Markov chain and 5000 dememorisation steps. Bonferroni correction for multiple  
12 testing was applied for each sample separately. The program FSTAT version 2.9.3.2  
13 (Goudet 2001) was used to test for deviations from genotypic equilibrium and  
14 calculate the allelic richness for each locus in each sample. The latter is a measure of  
15 the number of alleles independent of sample size (El Mousadik & Petit 1996). We  
16 tested for differences in allelic richness between samples 4, 6 and 7 from the three  
17 smallest fragments and all the others with the compare-groups option in FSTAT using  
18 1'000 permutations.

19 The significance of the genetic differentiation at different hierarchical levels was  
20 assessed with analyses of molecular variance (AMOVA) in ARLEQUIN using 16'000  
21 permutations. For all pairs of samples, we calculated pairwise  $F_{ST}$ -values and tested  
22 for significant differentiation with FSTAT. The latter analysis was performed with an  
23 exact *G*-test (Goudet *et al.* 1996). No correction for multiple testing was applied  
24 because procedures such as the sequential Bonferroni correction would be overly  
25 conservative in this situation, where a small number of type I errors can be tolerated  
26 without jeopardising the validity of our general conclusions. All *P*-values are given in  
27 Table 2.

28 The relationship between geographical and genetic distance in the large fragment 2  
29 was assessed with a Mantel test implemented in FSTAT using 2'000 permutations. To  
30 quantify the impact of geographical distance and roads on the amount of genetic  
31 differentiation observed between pairs of samples, a second Mantel test was  
32 performed on the entire data set from the Bernese study area. We tested the

1 significance of the partial regressions between pairwise  $F_{ST}$ -estimates and the  
2 following three explanatory variables: (i) geographical distance as measured on a  
3 map, (ii) number of roads and (iii) the age of the oldest road between any two sites in  
4 years. In matrix (ii), the highway was counted twice, because it was a dual  
5 carriageway of more than twice the width of the main roads with a narrow grass strip  
6 in the middle.

7 GeneClass version 2.0c (Piry *et al.* 2004) was used to compute the likelihood of each  
8 individual's genotype in each of the sampled populations based on the approach of  
9 Rannala & Mountain (1997). Each individual was assigned to the population where it  
10 had the highest likelihood. For each sample, we then determined the percentage of  
11 animals that were self-assigned based on that criterion, i.e. assigned to the population  
12 where they had been caught. The entire analysis was repeated with all samples from  
13 the same fragment pooled.

14 The average relatedness of individuals within samples was investigated using Identix  
15 version 1.0 (Belkhir *et al.* 2002). For each sample, we computed the average  
16 relatedness among all pairs of individuals as expressed by the identity index and  
17 compared this value to the expectation in a panmictic population. This null  
18 distribution was obtained by a resampling procedure implemented in Identix using  
19 1'000 permutations.

20 Spatial autocorrelation analysis was carried out with GenAlEx version 5.1 (Peakall &  
21 Smouse 2001). Individual-by-individual genetic distances were computed as  
22 described in Peakall *et al.* (2003) and implemented in GenAlEx. Geographical  
23 distance was determined as the linear distance in meters between pairs of sampling  
24 sites. We used the manual distance class option in GenAlEx with the following  
25 classes: <1m (containing all pairwise comparisons within sampling locations),  
26 <300m, <600m and so on. To compare the patterns of spatial autocorrelation in a  
27 continuous and a fragmented habitat, one analysis was based on all samples from  
28 fragment 2 (i.e. 2a-g), while a second analysis included one sample each from six  
29 different forest fragments (samples 2c, 3b, 4, 5, 6 and 7).

30 We carried out two tests to detect a potential bottleneck in the population from site 4,  
31 which had been reduced to an area of approximately 1 ha by the construction of the  
32 highway 30 years ago. The first analysis was based on the assumption that rare alleles  
33 are likely to be lost during a bottleneck and that, therefore, recently bottlenecked

1 populations would have fewer alleles at low ( $< 0.1$ ) than at medium frequencies  
2 (Luikart *et al.* 1998). A second test proposed by Garza & Williamson (2001) assumes  
3 that the number of alleles ( $k$ ) at a microsatellite locus will be reduced more strongly  
4 by a bottleneck than the difference in length ( $r$ ) between the longest and the shortest  
5 allele. Therefore, the quotient  $M = k/r$  is expected to be lower in a bottlenecked  
6 population than in a stable one. We calculated  $M$  for sample 4 as well as for three  
7 samples from large fragments (5, 2c and Spiez), where we would expect no recent  
8 bottleneck. The critical value of  $M$  for a given set of parameters was obtained with a  
9 program available from <http://www.pfeg.noaa.gov/tib/carlos.htm>. We assumed a step-  
10 wise mutation model with 10% larger mutations with a mean size of 3.5 repeat units  
11 as recommended in Garza & Williamson (2001) and  $\theta = 4N_e\mu = 20$ , which yielded a  
12 mean value for  $M$  in the same range as that observed in our three large populations.

13

#### 14 *Population density and mobility*

15 In order to assess the density and the mobility of *A. parallelepipedus*, capture-  
16 recapture experiments were carried out at sites 2c and 6 in areas adjacent to those used  
17 for tissue collection. At both locations, 256 plastic cups with a diameter of 6.5 cm  
18 were buried level with the soil surface in a grid of 16 x 16 traps at regular intervals of  
19 4 m. The traps were opened for two nights during warm and dry weather, emptied and  
20 then closed with plastic petri dishes for at least five nights. We carried out three  
21 trapping sessions at location 6 in July and August 2001 and eight at location 2c from  
22 May to August 2001. The captured beetles were individually marked with a  
23 characteristic pattern of one to ten points drilled into the surface of the elytrae  
24 (Mühlenberg 1993), and released at the trap where they had been caught.

25 To estimate population densities, the data were analysed with the program JOLLY  
26 version 01/24/91 (Hines 1988) based on the model by Jolly (1965), which allows for  
27 both death and immigration. For each recaptured individual, we further determined  
28 the linear distance between the traps where it had been caught and divided it by the  
29 number of nights between the relevant trapping periods. For each site, we calculated  
30 the mean distance and the 95% confidence limits based on the  $t$ -distribution. This  
31 method provides only a rough estimate of the minimum mobility of *A.*

32 *parallelepipedus* because (i) the animals probably do not walk from one trap to

1 another in a straight line and (ii) very mobile animals are more likely to leave the  
2 trapping area and will be underrepresented in the samples.

3

#### 4 *Computer simulations*

5 In a last step, we carried out computer simulations with the program EASYPOP version  
6 1.7.4 (Balloux 2001) to confirm that the observed divergence of the smallest  
7 population 4 could, indeed, be the result of isolation due to roads. In this case, it  
8 should be possible to generate comparable  $F_{ST}$ -values in simulations, in which we  
9 assume a short period of strong drift in one population and virtually no immigration  
10 from the other, larger population. To simplify the situation, we focused on only one  
11 pairwise comparison, namely between sites 4 and 5, which had been connected until  
12 the construction of the highway 30 years ago. The effective population size in  
13 fragment 5 was expected to be very large and, in the simulations, was held constant at  
14 10'000 individuals with a balanced sex ratio. This value was obtained by  
15 extrapolating an estimated census population density of 1000 individuals/ha to the  
16 minimum area of the fragment (40 ha) and assuming that  $N_e / N_c = 0.25$  (Frankham  
17 1995). It is clear that both of these values may not be very accurate and only a very  
18 rough estimate of the population size in the large fragment can be obtained. However,  
19 the exact value of this large  $N_e$  will have little effect on the outcome of the  
20 simulations, as the rate of random genetic drift in this population will be negligible.  
21 The effective size of the second population was varied between  $N_e = 50$  and  $N_e =$   
22 1000. To simulate the expected reduction in population size at site 4 in connection  
23 with the highway construction, we let  $F_{ST}$  reach an equilibrium value of zero by  
24 setting the migration rate to 0.99 for 970 generations, and then reduced the migration  
25 rate to 0, 0.0005 or 0.001, respectively, for another 30 generations. Each individual  
26 was assumed to have been characterized at 5 loci mutating at a rate of  $10^{-3}$  (Jarne &  
27 Lagoda 1996) according to a stepwise mutation model (Estoup & Cornuet 1999) with  
28 10% double-step mutations and a maximum of 30 possible allelic states. Preliminary  
29 tests showed that the choice of the parameters of the mutation model was not critical.  
30 After 1000 generations, a sample of 15 males and 15 females was drawn from each  
31 population, and the pairwise  $F_{ST}$ -value was calculated with FSTAT. The mean and the  
32 upper and lower quartiles of 100 replicates were computed for each set of parameter  
33 values.

## 1 **Results**

2

### 3 *Genetic diversity and linkage*

4 The five microsatellite loci were polymorphic in all samples, with the number of  
5 alleles ranging between 2 and 24. An allele frequency table is provided as an  
6 electronic appendix. Five of the tests for deviations from Hardy-Weinberg equilibrium  
7 indicated heterozygosity excess or deficit with  $0.01 < P < 0.05$  (Table 1). None of  
8 these tests remained significant after Bonferroni correction for loci within samples.  
9 Eleven out of a total of 160 tests for deviations from linkage equilibrium were  
10 significant at  $P < 0.05$ . As most of these significant values were observed between  
11 different pairs of loci, they were likely to be due to type I errors.

12 The allelic richness in the samples from the three smallest fragments was not  
13 significantly different from that in the rest of the study area (permutation test,  $P =$   
14 0.82), which indicated that the genetic variability was not significantly reduced in  
15 these populations.

16

### 17 *Population differentiation*

18 As expected, the largest pairwise  $F_{ST}$ -values were observed between the Swiss and the  
19 Belgian samples (Table 2). The genetic differentiation between the two Swiss  
20 locations of Bern and Spiez at about 40 km from each other was surprisingly low with  
21 an average pairwise  $F_{ST}$  of 0.011. Within the entire Bernese study area, we detected  
22 significant genetic differentiation with an overall  $F_{ST}$ -value of 0.004 (AMOVA,  $P =$   
23 0.049). Within fragment 2 (see Fig. 1), on the other hand, the global  $F_{ST}$ -value  
24 between the seven samples was not significantly different from zero (AMOVA,  $F_{ST} =$   
25 0.0008,  $P = 0.384$ ).

26 Of the 91 pairwise tests for population differentiation within the Bernese study area,  
27 21 were significant at the 5% level and seven at the 1% level (Table 2). Four of these  
28 tests significant with  $P < 0.01$  involved sample 4 from the smallest fragment in the  
29 highway exit loop (Fig.1). Remarkably, the average pairwise  $F_{ST}$  between this  
30 sampling location and all other Bernese sites was identical to the value observed  
31 between Bern and Spiez ( $F_{ST} = 0.011$ ). If sample 4 was omitted, the  $F_{ST}$  within the

1 rest of the Bernese study area was not significant anymore (AMOVA,  $F_{ST} = 0.0021$ ,  $P$   
2 = 0.171).

3 The Mantel test showed that there was no significant relationship between geographic  
4 and genetic distance within the large fragment 2 ( $P = 0.798$ ). The full analysis with all  
5 Bernese samples and three explanatory variables detected a significant partial  
6 regression between pairwise  $F_{ST}$ -values and the number of roads between study sites  
7 ( $P = 0.001$ ; Table 3), with this variable explaining 24.6% of the total variance.

8 Further, there was a significant negative relationship between  $F_{ST}$ -values and  
9 geographical distance ( $P = 0.008$ ), while the age of the oldest road between locations  
10 was not a significant variable ( $P = 0.727$ ). The model explained 29.3% of the total  
11 variance.

12 To assess how strongly the results of the Mantel test depended on the conspicuous  
13 sample 4 (see above), the analysis was repeated without this location. This gave  
14 similar if somewhat less pronounced results, indicating that the observed pattern was  
15 not solely due to sample 4 (Table 3). Again, the number of roads ( $P = 0.009$ ) and  
16 geographic distance between sites ( $P = 0.048$ ) were significant explanatory variable.  
17 The model explained 12.9% of the total variance.

18

### 19 *Individual-based analyses*

20 The percentage of self-assigned individuals was generally low (0 to 30%) and did not  
21 appear to be correlated with fragment size. If all samples from the same fragment  
22 were pooled, the highest percentages of self-assigned individuals were observed in the  
23 three smallest fragments 4, 6 and 7. This may indicate an increased level of  
24 differentiation between these populations and the rest of the study area. However, the  
25 percentages of self-assigned individuals were still very low (10 to 33%), indicating  
26 the limited power of assignment tests in this particular setting where genetic  
27 differentiation is low and only few microsatellite markers are available.

28 The relatedness analysis showed that the observed identity indices were not  
29 significantly different from the expectation in a panmictic population in any of the  
30 Bernese samples (data not shown).

31 The autocorrelation analysis, detected no significant spatial structure in any of the  
32 distance classes in fragment 2 (data not shown). In the analysis based on samples from

1 different fragments, significant positive autocorrelation was observed in the smallest  
2 distance class only, i.e. between individuals caught at the same sampling location ( $r =$   
3  $0.016$ ,  $P = 0.001$ ). Autocorrelation in this distance class remained significant if the  
4 analysis was repeated without the conspicuous sample 4 ( $r = 0.012$ ,  $P = 0.028$ ). These  
5 results indicate a patchy distribution of genotypes as would be expected in the case of  
6 restricted dispersal between fragments.

7

#### 8 *Detection of genetic bottlenecks*

9 Both tests provided no clear indication of a recent bottleneck in population 4. The  
10 analysis of the allele frequency distribution showed that, contrary to expectations after  
11 a bottleneck, most alleles occurred at low frequencies ( $< 0.1$ ; data not shown).  $M$  was  
12 somewhat lower in sample 4 ( $M = 0.6832$ ) than the average value from three large  
13 fragments, where no recent bottleneck would be expected ( $M = 0.7453$ ). However, for  
14 the given parameter values,  $M$  would have to be below the critical value of  $M_c \approx 0.62$   
15 to justify a rejection of the null hypothesis. Therefore, the reduction of  $M$  in sample 4  
16 was clearly not strong enough to suggest a recent reduction of the population size to a  
17 very low level.

18

#### 19 *Population density and mobility*

20 At location 2c, the mean density of *A. parallelepipedus* was estimated at 632  
21 individuals/ha (95% confidence interval: 332-935). The average minimum distance  
22 covered by the animals in one night was 1 m (95% c.i.: 0.71-1.29 m). At location 6,  
23 the density was considerably higher with 1707 individuals/ha (95% c.i.: 661-2748),  
24 and the animals covered a mean minimum distance of 2 m (95% c.i.: 1.65-2.35 m) per  
25 night. The fastest individual observed at the two sites walked 39.4 m in six nights,  
26 which was equivalent to 6.6 m/night. The observed densities are comparable to  
27 estimates of 1000-2000 individuals/ha of other studies (Loreau & Nolf 1993;  
28 Franceschini *et al.* 1997).

29

1 *Computer simulations*

2 Estimates comparable to the pairwise  $F_{ST}$  of 0.0142 observed between sites 4 and 5  
3 were obtained with different sets of parameters used in the simulations (Table 4). For  
4 example, the simulations with no migration and an effective population size of 500  
5 individuals resulted in an interval between the lower and upper quartile of 0.0104 –  
6 0.0200. Alternatively,  $F_{ST}$ -values in the same order of magnitude were obtained in the  
7 simulations with  $N_e = 200$ , but allowing for some migration ( $0.0005 \leq m \leq 0.001$ ).

8

## 1 **Discussion**

### 2 *Habitat fragmentation due to roads?*

3 In an ideal study on the effects of habitat fragmentation, we would possess data on the  
4 genetic population structure of a species in a certain area before and some time after  
5 the construction of roads, to exclude the possibility that the observed differentiation  
6 was already present in the unfragmented habitat. Such data are generally not  
7 available, but can be approximated by working at a very small scale where no genetic  
8 population structure would be expected in a homogenous habitat. In such a setting, the  
9 impact of roads will not be mingled with the impact of geographical distance or the  
10 potential barrier effect of other landscape elements such as agricultural or urban areas.  
11 It is also important to choose a study area, where the roads do not follow conspicuous  
12 topographic elements, which might have represented older, natural barriers to gene  
13 flow. Finally, it is possible to formulate precise hypotheses on the patterns of  
14 differentiation that should be observed, if genetic differentiation were indeed the  
15 result of isolation due to roads. Thus, the impact of recent fragmentation should first  
16 be detectable in the smallest habitat fragments, while larger populations may remain  
17 undifferentiated even if they are completely isolated from each other. This is a  
18 consequence of the inverse relationship between the rate of population divergence and  
19 effective population size discussed in the introduction. If such a pattern is indeed  
20 observed this will provide further support for the hypothesis that roads are important  
21 factors determining the intensity of gene flow between local populations.

22 Our results supported the hypothesis that no genetic differentiation would be observed  
23 at such a local scale in a continuous forest. The overall  $F_{ST}$ -value within the large  
24 fragment 2 (Fig. 1) was not significantly different from zero (AMOVA,  $P = 0.384$ )  
25 and no significant association between genetic and geographical distance was detected  
26 ( $P = 0.798$ ). As hypothesised, the Mantel test based on all samples detected a highly  
27 significant increase of pairwise  $F_{ST}$ -values with the number of roads between  
28 sampling locations (Table 3). Quite surprisingly, this analysis also showed a  
29 significant negative association between geographical distance and  $F_{ST}$ -values. This  
30 indicated that genetic differences tended to be larger between locations which were  
31 geographically close than between more distant sites. This rather surprising result  
32 could be largely due to sample 4 from the smallest fragment, which was significantly  
33 differentiated from many other sites, but was geographically close to several sampling

1 locations in other forest fragments. If this sample was omitted from the analysis, the  
2 negative association between geographical and genetic distance became weaker but  
3 remained significant ( $P = 0.048$ ), indicating that other locations also contributed to  
4 this pattern.

5 As mentioned above, the most conspicuous sample in terms of number of significant  
6 test for differentiation was clearly sample 4 from the highway exit loop, which was  
7 significantly differentiated from nine of the 13 other Bernese locations. Furthermore,  
8 the lowest  $P$ -values among the Bernese sites were observed in pairwise comparisons  
9 involving this particular sample (Table 2). As stated before, conventional corrections  
10 for multiple testing, such as sequential Bonferroni correction, are overly conservative  
11 in this situation where some type I errors can be tolerated. If several tests involving a  
12 particular sample give  $P$ -values below  $\alpha$ , it is quite unlikely that these results are all  
13 spurious (see e.g. Moran 2003). Thus, the high number of significant pairwise  
14 comparisons involving sample 4 suggests that a considerable fraction of the genetic  
15 differentiation within the Bernese study area measured by a significant overall  $F_{ST}$  ( $P$   
16 = 0.049) is due to this particular sample. This result is in perfect agreement with our  
17 expectation that the impact of fragmentation due to roads should first be detectable in  
18 the smallest forest patch.

19 However, the significant effects of roads on population structure were not solely  
20 caused by this one conspicuous sample. If site 4 was omitted from the analysis, the  
21 Mantel test still detected a significant increase of pairwise  $F_{ST}$ -values with the number  
22 of roads between locations (Table 3). Due to the inverse relationship between  
23 effective population size and genetic drift, the smaller fragments were expected to  
24 diverge more quickly than the larger ones. Indeed, samples 6 and 7 from the second  
25 and third smallest fragments were significantly differentiated from 38% of the other  
26 Bernese locations. Although this value was lower than that observed for fragment 4  
27 (69%), it was considerably higher than the percentage of significant between-fragment  
28 comparisons observed for the remaining fragments (0 - 22%).

29 Within fragments, three out of 22 pairwise comparisons were significant at the 0.05  
30 level and two at the 0.01 level (Table 2). Although some type I errors are likely, this  
31 result indicates that factors other than roads may be responsible for some genetic  
32 structure between locations. For example, local relatedness structure resulting from  
33 patchy colonization and associated groups of relatives could lead to the observed

1 genetic differentiation within fragments. However, the individual-based analyses did  
2 not provide any clear indication that such factors contributed substantially to the  
3 observed population structure. The results of these analyses, in particular the spatial  
4 autocorrelation analysis, are consistent with our general conclusions.

5

#### 6 *Comparison between species*

7 Our findings provide good support for the hypothesis that roads led to the isolation of  
8 *A. parallelepipedus* populations in different forest fragments. These findings are  
9 concordant with the results previously obtained for *Carabus violaceus* in the same  
10 study area (Keller & Largiadèr 2003a), although considerably less pronounced. In *C.*  
11 *violaceus*, the number of roads between locations explained 44% of the variation in  
12 pairwise  $F_{ST}$ -values, and the impact of recent isolation was already detectable in  
13 larger forest patches, particularly in fragment 3. For example, 92% of the pairwise  
14 comparisons between samples 3a and 3b and all other locations were significant at the  
15 0.05 level. *C. violaceus* was absent from the next smallest fragments 6 and 7 (Fig. 1).  
16 In fragment 4 in the highway exit loop, a total of only fifteen individuals had been  
17 caught in two years. In spite of this small sample size, this study site was significantly  
18 differentiated from five of the seven other locations. Although different microsatellite  
19 loci were used in *C. violaceus* it is unlikely that the stronger impact of roads observed  
20 in this species was solely the result of a higher power to detect population  
21 differentiation.

22 A less pronounced fragmentation effect of roads on *A. parallelepipedus* could relate to  
23 the higher population densities observed in this species (632 and 1707 individuals/ha;  
24 this study) as compared to *C. violaceus* (88 individuals/ha; I. Keller, unpublished  
25 data). If population density is at least roughly proportional to the effective population  
26 size, the process of genetic differentiation due to random drift will be much slower in  
27 *A. parallelepipedus*. Of course, potential differences in dispersal ability between the  
28 two species may also have contributed to the observed pattern.

29 The amount of genetic differentiation was generally quite low in *A. parallelepipedus*,  
30 even at a larger geographical scale. In particular, the average  $F_{ST}$ -value of 0.013  
31 observed between the Bernese samples and Spiez was surprisingly low. We can only  
32 speculate about possible reasons for this result. For example, it is conceivable that the

1 population from Spiez and most of the Bernese populations are so large that the rate  
2 of genetic drift will be too low to produce detectable differences even over thousands  
3 of generations. As expected, the highest  $F_{ST}$ -values of around 0.1 were observed  
4 between Switzerland and Belgium (Table 2). These values were considerably lower  
5 than the estimates obtained in *C. violaceus* ( $F_{ST} = 0.4$ ; Keller & Largiadèr 2003a).

6

#### 7 *Estimating road effects using computer simulations*

8 The computer simulations showed that  $F_{ST}$ -values in the order of magnitude observed  
9 between samples 4 and 5 ( $F_{ST} = 0.014$ ) could be obtained in the case of no gene flow  
10 and an effective population size ( $N_e$ ) of about 500 individuals in the smaller  
11 population (Table 4). Similar results could be obtained under a different scenario with  
12 a lower value for  $N_e$  (= 200) but allowing for some gene flow. Our recapture data on  
13 mobility indicated that *A. parallelepipedus* was able to cover distances of a few  
14 metres in one night and would, theoretically, be able to cross a main road in a  
15 reasonable amount of time. In his extensive mark-recapture study, Mader (1984)  
16 showed that such road crossings were indeed possible but extremely rare in *A.*  
17 *parallelepipedus*, supporting the hypothesis of very little gene flow. The results of the  
18 simulations are in good agreement with our field data on census population sizes,  
19 which showed that more than 1500 individuals could occur in an area of 1 ha. As the  
20 effective population size is generally smaller than the census size (Frankham 1995),  
21 values for  $N_e$  of a few hundred individuals appear to be reasonable.

22 The simulation result for  $N_e = 1000$  and  $m = 0$  (Table 3) also provides an expectation  
23 of the pairwise  $F_{ST}$ -value between sites 5 and 6. The census population size in  
24 fragment 6 was estimated at 1700 individuals/ha in the capture-recapture analysis. If  
25 this value is extrapolated to the area of the fragment (ca. 2.5 ha), we obtain a total  
26 census population size of 4250 individuals and an estimated effective population size  
27 of just above 1000 individuals, assuming that  $N_e / N_c = 0.25$ . The simulations with  $N_e$   
28 = 1000 and  $m = 0$  resulted in a mean  $F_{ST}$  of 0.0088 and a range of 0.0047 – 0.0122,  
29 which is in perfect agreement with the observed value of 0.0098 (Table 2).

30

31 The finding that the simulations with realistic parameters for  $N_e$ , migration rate and  
32 divergence time (i.e. the time since the construction of roads) yielded  $F_{ST}$ -values  
33 compatible with the observed ones supports our hypothesis that the genetic structure

1 was indeed due to the roads and had not already been present before their  
2 construction.

3

#### 4 *Population bottlenecks*

5 The failure to detect any signs of a recent population bottleneck in sample 4 could be  
6 due to the low power of such tests when a limited number of loci are used. The  
7 method of Luikart *et al.* (1998), for example, has a power of 80% to detect a  
8 bottleneck of < 20 individuals when 8-10 loci are used, which is twice the number of  
9 loci available for *A. parollepipedus*. The second approach will also lead to more  
10 reliable estimates as the number of markers increases, because the variance of  $M$   
11 between loci can be quite large (Garza & Williamson 2001). A much more likely  
12 explanation for the failure to detect a bottleneck, however, is that  $N_e$  was not reduced  
13 to extremely low levels by habitat fragmentation. Thus, our computer simulations  
14 suggested an effective population size of a few hundred individuals in fragment 4.  
15 Furthermore, our capture-recapture data showed that the densities reached by the  
16 species were so high that a very extreme reduction of the population size to only a few  
17 individuals at site 4 was unlikely, unless the habitat had been completely destroyed  
18 during the construction of the highway. The more severe a reduction in population  
19 size, however, the more likely it is to be detected (Luikart *et al.* 1998).

20

1    **Conclusions**

2    Our results suggested that habitat fragmentation due to roads could lead to strong  
3    isolation of *A. parallelepipedus* populations in different forest fragments. Due to the  
4    often large population sizes, the probability of local extinctions may be relatively low.  
5    If they do occur, however, such extinctions are serious, because the recolonisation of  
6    suitable but isolated habitats may be virtually impossible because of the flightlessness  
7    and consequently low dispersal abilities of *A. parallelepipedus*. Indeed, previous  
8    studies indicated that isolation might have a considerable effect on the distribution of  
9    this abundant but poorly dispersing species. For example, the area inhabited by *A.*  
10    *parallelepipedus* in the Netherlands has decreased during the last century, while  
11    species with better dispersal abilities did not show such a trend (Turin & den Boer  
12    1988). In Germany, *A. parallelepipedus* was found significantly more often in ancient  
13    forests than in more recent ones, which was ascribed to the low mobility of the  
14    species (Assmann 1999). In conclusion, our study suggests that the isolating effect of  
15    roads should not be underestimated even in high-density species. This further  
16    underlines the importance of efforts in conservation practice to increase the  
17    connectivity among isolated habitats.

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1 **Author Information Box**

2 This study is part of the PhD work of Irene Keller on the effects of habitat  
3 fragmentation on two species of ground beetles. Wolfgang Nentwig is professor of  
4 ecology. Carlo R. Largiadè is interested in the conservation genetics of fish and  
5 invertebrate species.

1 **Figure Legend**

2

3 Fig. 1: Location of the sampling sites at Bremgartenwald, Bern. r1 = main road  
4 (present width: 9 m; min. age at the time of sampling: 130 yrs), r2 = main road  
5 (width: 8 m; age: 87 yrs), r3 = highway and highway exit between sites 4 and 5 (width  
6 of highway: 30 m; age: 31 yrs).

1 Table 1: Genetic variability at five microsatellite loci in 16 samples from *A.*  
2 *parallelepipedus*. Listed are number of alleles ( $N_A$ ), observed ( $H_O$ ) and expected ( $H_E$ )  
3 heterozygosities and number of individuals scored in each sample ( $N$ ). For each locus,  
4 repeat motive and EMBL accession nos are indicated. Below the table,  $P$ -values are  
5 given for significant deviations from Hardy-Weinberg equilibrium.  
6

		<b>locus name</b>				
		repeat motif				
		accession #				
		<b>APA05103</b>	<b>APA06272</b>	<b>APA12499</b>	<b>APA14224</b>	<b>APA14449</b>
		<b>CMPG</b>	<b>CMPG</b>	<b>CMPG</b>	<b>CMPG</b>	<b>CMPG</b>
		(CATA) <sub>n</sub>	(CA) <sub>n</sub>	(CATA) <sub>n</sub>	(GT) <sub>n</sub> AT(GT) <sub>n</sub>	(TG) <sub>n</sub>
<b>sample</b>		AJ510193	AJ510194	AJ510192	AJ510195	AJ510196
<b>1</b>	$N_A$	4	4	2	5	21
	$N = 30$	$H_O$	0.586 <sup>a</sup>	0.567	0.310	0.533
		$H_E$	0.687	0.606	0.296	0.503
<b>2a</b>	$N_A$	3	4	2	8	17
	$N = 30$	$H_O$	0.552 <sup>b</sup>	0.467 <sup>c</sup>	0.233	0.467
		$H_E$	0.595	0.644	0.259	0.514
<b>2b</b>	$N_A$	3	7	2	4	16
	$N = 15$	$H_O$	0.533	0.800	0.133	0.533
		$H_E$	0.632	0.685	0.297	0.621
<b>2c</b>	$N_A$	3	6	2	5	19
	$N = 30$	$H_O$	0.600	0.667	0.100	0.567
		$H_E$	0.615	0.637	0.186	0.640
<b>2d</b>	$N_A$	3	4	2	5	18
	$N = 18$	$H_O$	0.667	0.778	0.111	0.611
		$H_E$	0.637	0.635	0.108	0.603
<b>2e</b>	$N_A$	4	5	2	6	20
	$N = 32$	$H_O$	0.548	0.633	0.344	0.688
		$H_E$	0.602	0.669	0.365	0.673
<b>2f</b>	$N_A$	3	5	3	7	19
	$N = 32$	$H_O$	0.688	0.531	0.313	0.563
		$H_E$	0.636	0.641	0.299	0.574
<b>2g</b>	$N_A$	3	6	2	6	24
	$N = 33$	$H_O$	0.613	0.788	0.212	0.485
		$H_E$	0.613	0.704	0.220	0.546
<b>3a</b>	$N_A$	3	5	4	8	20
	$N = 30$	$H_O$	0.593	0.586	0.333	0.600
		$H_E$	0.660	0.586	0.403	0.632
<b>3b</b>	$N_A$	3	5	4	6	19
	$N = 30$	$H_O$	0.633	0.767	0.433	0.433
		$H_E$	0.636	0.714	0.421	0.428

<b>4</b> <i>N</i> = 30	<i>N<sub>A</sub></i>	3	6	2	6	19
	<i>H<sub>O</sub></i>	0.704	0.533	0.233	0.500	0.900
	<i>H<sub>E</sub></i>	0.581	0.508	0.332	0.603	0.938
<b>5</b> <i>N</i> = 30	<i>N<sub>A</sub></i>	4	6	2	5	22
	<i>H<sub>O</sub></i>	0.565	0.621	0.367	0.467	0.926
	<i>H<sub>E</sub></i>	0.676	0.615	0.332	0.438	0.945
<b>6</b> <i>N</i> = 30	<i>N<sub>A</sub></i>	3	5	2	7	21
	<i>H<sub>O</sub></i>	0.464	0.500	0.267	0.733	0.931
	<i>H<sub>E</sub></i>	0.576	0.647	0.315	0.706	0.947
<b>7</b> <i>N</i> = 30	<i>N<sub>A</sub></i>	3	5	3	7	22
	<i>H<sub>O</sub></i>	0.593	0.733 <sup>d</sup>	0.367	0.533	0.929
	<i>H<sub>E</sub></i>	0.667	0.649	0.389	0.604	0.948
<b>Spiez</b> <i>N</i> = 30	<i>N<sub>A</sub></i>	3	6	2	5	21
	<i>H<sub>O</sub></i>	0.536	0.517	0.300	0.367	1.000
	<i>H<sub>E</sub></i>	0.618	0.486	0.332	0.469	0.955
<b>Belgium</b> <i>N</i> = 20	<i>N<sub>A</sub></i>	3	3	2	3	10
	<i>H<sub>O</sub></i>	0.300	0.550 <sup>e</sup>	0.200	0.550	0.737
	<i>H<sub>E</sub></i>	0.387	0.665	0.304	0.535	0.734

<sup>a</sup> *P* = 0.047    <sup>b</sup> *P* = 0.037    <sup>c</sup> *P* = 0.046    <sup>d</sup> *P* = 0.018    <sup>e</sup> *P* = 0.011

1

2

1 Table 2: Below diagonal: pairwise  $F_{ST}$ -estimates. Above diagonal:  $P$ -values of  $G$ -tests for pairwise differentiation. Bold =  $0.01 < P < 0.05$ ;  
 2 shaded =  $P < 0.01$ . Sp = sample from Spiez; Be = sample from Belgium.

3  
 4

	Bernese samples													Sp	Be	
	1	2a	2b	2c	2d	2e	2f	2g	3a	3b	4	5	6	7		
1		.0579	.3783	.3250	.5267	.1238	.2888	.7450	.5971	.4833	.1338	.5900	.3054	.5071	.2817	.0004
2a	-.0016		.0692	.0592	.1100	.0746	.1029	.0025	.1354	.1167	.0017	.1938	<b>.0333</b>	.0796	.2904	.0004
2b	-.0022	.0043		.4579	.3067	.3017	.8108	.4279	.1513	.3263	.3138	.3792	.1554	.0983	.3171	.0004
2c	.0024	.0041	-.0084		.1075	<b>.0263</b>	.1600	.4883	.6254	<b>.0317</b>	<b>.0208</b>	.7400	.2142	<b>.0225</b>	.1017	.0004
2d	-.0042	-.0040	.0012	-.0052		.1950	.7563	.7975	.5171	.4675	.1717	.4517	.4904	.1904	.2525	.0004
2e	.0040	.0052	-.0044	.0067	.0087		.1904	.0079	.6008	.0654	.0025	.1050	<b>.0388</b>	<b>.0429</b>	<b>.0300</b>	.0004
2f	-.0047	-.0038	-.0015	-.0035	-.0116	.0047		.1775	.6613	.3338	<b>.0275</b>	.2142	<b>.0488</b>	<b>.0308</b>	<b>.0213</b>	.0004
2g	-.0005	.0031	-.0010	-.0022	-.0071	.0110	-.0025		.1888	.1033	.1608	.5392	.5967	.1808	.0017	.0004
3a	-.0033	.0008	.0032	.0006	-.0008	-.0040	-.0056	.0036		.3942	<b>.0292</b>	.5750	.4579	.7333	.1254	.0004
3b	-.0012	.0005	.0097	.0191	.0060	.0064	.0018	.0050	.0048		.0038	.5004	.0083	<b>.0292</b>	<b>.0179</b>	.0004
4	.0071	.0123	.0009	.0037	.0045	.0187	.0046	.0108	.0123	.0260		.0075	<b>.0383</b>	<b>.0104</b>	.0021	.0004
5	-.0085	-.0047	.0054	.0053	-.0035	.0058	-.0057	.0020	-.0030	-.0037	.0142		.1342	.0883	.5179	.0004
6	.0097	.0036	.0071	-.0027	-.0029	.0095	-.0007	-.0017	-.0018	.0173	.0080	.0098		.4942	.0838	.0004
7	.0006	.0032	.0126	.0122	.0084	.0048	.0040	.0054	-.0085	.0041	.0228	.0038	.0029		.0521	.0004
Sp	-.0029	.0055	.0034	.0149	.0103	.0091	.0090	.0241	.0069	.0165	.0142	-.0007	.0256	.0180		.0004
Be	.1065	.1123	.0914	.1108	.1277	.0814	.1168	.0934	.0929	.1050	.1492	.1163	.1097	.0870	.1302	

5

Table 3: Results of the Mantel test: partial regression coefficients ( $b_{Yj}$ ) between pairwise  $F_{ST}$ -values and geographic distance, number of roads and age of roads, respectively,  $P$ -values for  $b_{Yj}$  and determination coefficients (det).

	all samples			without sample 4		
	$b_{Yj}$	$P$ -value	det (%)	$b_{Yj}$	$P$ -value	det (%)
geographic distance	$-7 \times 10^{-6}$	<b>0.008</b>	5.6	$-5 \times 10^{-6}$	<b>0.048</b>	2.6
number of roads	0.003	<b>0.001</b>	24.6	0.002	<b>0.009</b>	10.6
age of roads	$-7 \times 10^{-6}$	0.727	-0.9	$-4 \times 10^{-6}$	0.848	-0.3
% of variance explained by the model			29.3			12.9

Table 4:  $F_{ST}$ -values (mean and range between lower and upper quartile) obtained in computer simulations with the specified values for effective population size ( $N_e$ ) and migration rate ( $m$ ). 100 replicates were analysed for each set of parameter values.

$N_e$	$m$	mean $F_{ST}$	range
50	0	0.1267	0.0950 – 0.1528
200	0	0.0366	0.0243 – 0.0438
500	0	0.0155	0.0104 – 0.0200
1000	0	0.0088	0.0047 – 0.0122
200	0.0005	0.0187	0.0119 – 0.0235
200	0.0010	0.0107	0.0048 – 0.0157

