Genetic effects of chronic habitat fragmentation in a wind-pollinated tree

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Habitat fragmentation poses a serious threat to plants through genetic changes associated with increased isolation and reduced population size. However, the longevity of trees, combined with effective seed or pollen dispersal, can enhance their resistance to these effects. The European beech (*Fagus sylvatica*) dominates forest over large regions of Europe. We demonstrate that habitat fragmentation in this species has led to genetic bottlenecks and the disruption of the species' breeding system, leading to significantly elevated levels of inbreeding, population divergence, and reduced genetic diversity within populations. These results show that, in contrast with the findings of previous studies, forest fragmentation has a negative genetic impact, even in this widespread, wind-pollinated tree. The identification of significant effects of forest fragmentation in beech demonstrates that trees are not at reduced risk from environmental change. This should be accounted for in the management of remaining natural and seminatural forest throughout the world.

F-statistics | forest fragmentation | inbreeding | isolation by distance | population genetics

The fragmentation of a species' habitat into smaller and more isolated remnants represents a potentially serious threat to biodiversity (1). Theory predicts that habitat fragmentation should lead to the disruption of plant breeding systems, leading to increased inbreeding and population differentiation and the erosion of genetic variability within populations (1–6). In the short term, increased inbreeding can lower individual fitness and reduce population viability, thereby increasing the extinction risk of individual populations (1, 2, 4). In the long term, reduced genetic diversity may limit the species' evolutionary potential to respond to environmental change (1, 7, 8). Individual forest fragmentation studies have provided only limited support for these theoretical predictions (4–6, 9–16). This limited support has led to the belief that tree species [particularly those that are wind-pollinated (5, 6, 9, 12)] are especially resistant to the effects of habitat fragmentation and are therefore at low risk from environmental change (3).

Although there are numerous studies of the effects of forest fragmentation in tropical regions (4), relatively little attention has been paid to the genetic consequences of forest fragmentation on temperate trees (10), despite the fact that historic deforestation of many temperate regions has equaled the magnitude, if not the rate, of current tropical deforestation (5) and often preceded it by many hundreds of years (4, 5, 9, 10). However, it has been argued that it is widespread, community-dominant species that are most at risk from habitat fragmentation (8), a classification that describes many forest-forming trees of temperate and boreal regions.

Investigation of the effects of forest fragmentation has been hampered by poor replication (due to few remaining fragments) and the lack of comparative data from prefragmentation populations (3, 4). Studies have sometimes been forced to rely on comparison of forest fragments with continuous forest in different areas of the species' geographic range (9–11, 16), despite the fact that underlying geographical patterns of population divergence and genetic diversity (5, 17, 18) may make such comparison problematic. Many studies assess fragmentation that has occurred only within the last 10–200 years (4, 6, 12) and, given the longevity of most tree species, are likely to be too recent to provide a clear picture of the genetic fate of the studied populations (4, 13).

Where fragmented and continuous forest areas coexist, the historic fragmentation of temperate forests provides the opportunity to assess the effects of chronic habitat fragmentation and to test the theoretical predictions outlined above. Here, we report the effects of >600 years of habitat fragmentation on the genetic diversity and population genetic structure of European beech (*Fagus sylvatica*) in Catalonia, northeast Spain. We find significant and ongoing effects of habitat fragmentation in this species. Our results demonstrate that tree species are at significant risk from habitat fragmentation, a finding that has important implications for the management of remaining natural and seminatural forest throughout the world.

Results

We genotyped individuals at six highly variable microsatellite loci. These loci gave an average of between 2.93 (±0.07) and 12.64 (±0.56) alleles per locus per sample (means are followed by SE in parentheses). Observed and expected mean heterozygosities per sample ranged from 0.563 (±0.078) and 0.698 (±0.077) to 0.723 (±0.080) and 0.729 (±0.057). Tests of Hardy–Weinberg equilibrium (HWE) per locus on each of the 14 samples showed that 6 of 84 cases showed a significant departure from HWE [$F_{is} \neq 0$, $\alpha = 0.05$ after Bonferroni (19) correction, 1,680 randomizations]; these did not cluster by locus or sample.

Levels of inbreeding (positive F_{is}) were analyzed by testing departure from HWE calculated over all loci within samples. Six of the seven fragmented forest samples showed significant levels of inbreeding [$\alpha = 0.05$ after Bonferroni (19) correction, 1,680 randomizations], whereas none of the continuous forest samples deviated from HWE. Levels of inbreeding in fragmented forest samples were twice as high as levels of inbreeding in continuous forest ($P_{\text{one tailed}} = 0.0028$, 2,500 permutations) (Table 1). However, whereas the 95% confidence interval (C.I.) indicates that inbreeding in the forest fragments is significantly raised above 0 ($F_{is} = 0.127$, 95% C.I. = 0.055–0.223), the confidence interval for F_{is} in continuous forest includes 0, confirming that no significant inbreeding within samples occurs in this group ($F_{is} = 0.062$, 95% C.I. = −0.003–0.157).

Genetic differentiation (F_{st}) between samples within each group was three times higher for forest fragments when compared with samples from continuous forest ($P_{\text{one tailed}} = 0.0016$, 2,500 permutations: $F_{st} = 0.029$, 95% C.I. = 0.024–0.039; continuous forest: $F_{st} = 0.010$, 95% C.I. = 0.004–0.017) (Table

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Abbreviations: HWE, Hardy–Weinberg equilibrium; ha, hectares.

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Table 1. Population genetic structure and genetic diversity compared between forest fragments and continuous forest

Parameter	Forest fragments	Continuous forest	*P*
Number of bottlenecked populations	5	0	**0.0105**
Inbreeding coefficient, F_{is}	0.127 (0.044)	0.062* (0.042)	**0.0028**
Genetic differentiation, F_{st}	0.029 (0.004)	0.010 (0.003)	**0.0016**
Number of rare alleles absent	23	5	**0.00004**
Allelic richness	8.257 (0.395)	9.335 (0.377)	**0.0044**
Observed heterozygosity, H_O	0.611 (0.014)	0.647 (0.013)	0.062
Gene diversity, H_S	0.700 (0.009)	0.691 (0.008)	0.75

Comparisons are between seven forest fragment samples and seven samples from continuous forest. Group means are followed by standard error in parentheses. Absence of rare alleles is calculated as the number of alleles present with frequency <0.05 over all populations that is absent from pooled fragments or continuous forest. Statistically significant comparisons are highlighted in bold type, and the marginally significant comparison for H_O is underlined.

*F_{is} was not significantly different from 0 in continuous forest (see text).

1). Pairwise population differentiation tests carried out by using the log-likelihood statistic *G* (20) (not assuming HWE) showed that whereas all possible population pairs (21 combinations) within the fragmented forest were significantly differentiated ($\alpha = 0.05$ after Bonferroni correction, 420 permutations), significant differentiation was found between only 12 of the 21 population pairs analyzed in the continuous forest.

The program BOTTLENECK (21) was used to test the hypothesis that the forest fragments have resulted from a recent reduction in effective population size, as opposed to being historically small populations. Recent bottlenecks were detected in five of the seven forest fragments ($P < 0.05$, Wilcoxon sign-rank test) and none of the continuous forest samples ($P > 0.05$). Consequently, population bottlenecks were associated with forest fragments rather than continuous forest ($P_{\text{one tailed}} = 0.0105$, Fisher's exact test) (Table 1).

To differentiate between minor but real population structure and artifacts due to noise-related sampling errors (22), we calculated Nei's (23) genetic distance between all population pairs within each group and tested its relationship with the corresponding geographic distance between populations using a Mantel test (24). A significant relationship occurred between genetic and geographic distance in forest fragments ($r = 0.401$, $P = 0.0422$, 30,000 randomizations), with the regression model [reduced major axis regression (24)] explaining 16% of the variance between them (Fig. 1). In contrast, genetic distance was not related to geographic distance in continuous forest ($r = 0.097$, $P = 0.320$, 30,000 randomizations).

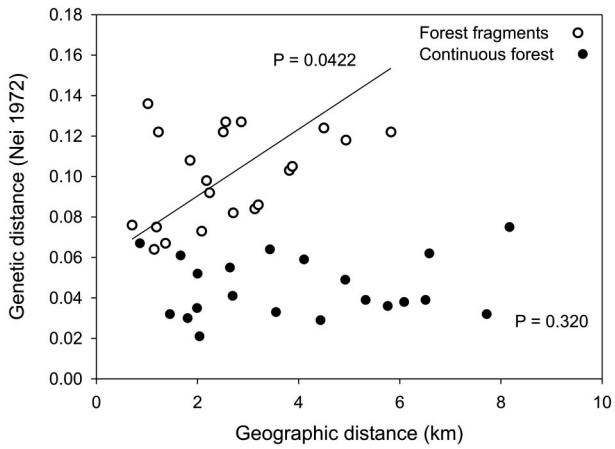

Fig. 1. Genetic distance (23) based on six microsatellite loci, vs. linear geographic distance for all possible pairwise combinations of *F. sylvatica* samples within each group. A significant positive correlation is observed for forest fragments only: regression analysis $y = 0.0574 + 1.65 \times 10^{-5}x$, $r = 0.401$, $P = 0.0422$. A significant positive correlation indicates that samples are spatially genetically structured, with isolation by distance playing an important role.

Genetic diversity of the populations in each group (fragmented forest versus continuous forest) was assessed by using the measure of allelic richness and the proportion of rare alleles, observed heterozygosity (H_O), and within-population gene diversity (H_S). Genetic diversity was high in both groups. Rare alleles [those with a frequency of ≤0.05 (14)] accounted for 71 (70%) of the total 102 alleles detected over all samples. They were less likely to occur in forest fragments: 23 were absent from the pooled fragmented forest samples, whereas only 5 were absent from the continuous forest ($\chi^2 = 14.41$, df = 1, $P = 0.00004$, χ^2 test). Allelic richness was 12% lower in fragmented forest samples than in continuous forest (8.257 fragments; 9.335 continuous forest, $P_{\text{one tailed}} = 0.0044$, 2,500 permutations). Observed heterozygosity was lower in fragmented populations, although the statistical significance of the difference was marginal ($H_O = 0.611$ fragments; 0.647 continuous forest, $P_{\text{one tailed}} = 0.062$). There was no significant difference in gene diversity (H_S) between the two groups ($P_{\text{one tailed}} = 0.75$) (Table 1).

In an exploratory study, we compared levels of genetic diversity and population genetic structure in old and young trees within two of the forest fragments that we investigated. In both fragments, allelic richness was lower and inbreeding was higher in the more recently established trees. However, inbreeding among old trees was significantly greater than zero in only one fragment (allelic richness: F_3 old = 10.44, F_3 young = 8.59, F_4 old = 9.10, F_4 young = 8.27; F_{is}: F_3 old = 0.071, F_3 young = 0.105,† F_4 old = 0.118,† F_4 young = 0.159†). F_{st} was slightly higher when young samples were compared between fragments than when the comparison was made between old-tree samples [F_{st}: young = 0.018 (0.006–0.035), old = 0.016 (0.006–0.029), 95% confidence limits are given in parentheses].

Discussion

Our results show a significant effect of habitat fragmentation on European beech over the last 600 years. In continuous forest, no spatial genetic structure is evident from isolation-by-distance patterns, and population differentiation is very low, indicating that at the spatial scale of our study, the continuous forest area is subject to panmictic breeding (25). Of the 21 pairwise population differentiation tests, the 12 significant tests are more likely to result from the great power of the log likelihood statistic *G* to

†$F_{is} \neq 0$, $\alpha = 0.05$ after Bonferroni correction, 480 randomizations; F_3 and F_4 denote the sample number.

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detect very small differences in allele frequencies between the continuous forest samples than from any biologically meaningful genetic structure (22). In contrast, we detected significant isolation-by-distance between forest fragments together with increased F_{st} and significant population differentiation between all pairwise forest fragment comparisons. The influence of gene flow between fragments therefore declines relative to the effects of genetic drift as the physical distance between them increases (25). This pattern indicates that fragmentation of the beech forest in this region has led to the breakup of the panmictic breeding system observed in the neighboring area of continuous forest.

Although a significant reduction in the genetic diversity of forest fragments was detected by using the measure of allelic richness, gene diversity (H_S) did not differ between the two groups of samples. Allelic richness is more sensitive to reductions in population size than is gene diversity because of the preferential elimination of rare alleles that contribute little to heterozygosity (21, 26). Because gene diversity may take many generations to reach equilibrium after a reduction in population size (21, 27), allelic richness may more accurately reflect current levels of genetic diversity within the fragmented populations.

The nonequilibrium status of the fragmented forest populations is highlighted by the detection of recent bottlenecks in these samples (Table 1). The test implemented in BOTTLENECK relies on the detection of the transient gene diversity excess that occurs after a reduction in population size before gene diversity has reached the value predicted at equilibrium from the number of alleles within the sample (21). The detection of bottlenecks by using this method indicates that although gene diversity is currently similar in continuous forest and forest fragments (Table 1), it may decline in the forest fragments over future generations. The detection of recent bottlenecks also corroborates historical evidence that the forest fragments were once part of a much larger population (28, 29).

The isolation-by-distance patterns shown in Fig. 1 demonstrate that forest fragmentation in European beech has resulted in gene flow between fragments being reduced below the levels observed in prefragmentation populations. However, given the size of the remaining fragments [21–52 hectares (ha)], it is unlikely that reduced gene flow alone can explain the additional genetic effects (elevated inbreeding, increased differentiation, and reduced diversity) (Table 1) that we report here (3, 8). These alterations to genetic structure and diversity are more likely to result from the increased genetic isolation of the remaining fragments acting in concert with a substantial reduction in effective population size (1, 2). It is possible that the deforestation of the southwest ridge of the Montseny Mountains was more severe than is currently observed and left only scattered small forest patches remaining. These small fragments would have subsequently increased in size and merged to form the larger fragments that we see today. The genetic bottlenecks experienced by these fragmented populations would cause the independent loss of (predominantly rare) alleles from each fragment, thereby increasing population divergence. The elevated inbreeding that we report would result from both mating between related individuals in the bottlenecked populations and a potential Wahlund effect caused by significant genetic structure remaining within the fragments we sampled.

Given the proximity of the fragmented populations to each other and the continuous forest, it could be argued that the fragments should be gaining diversity and becoming less inbred over time (6). However, in both fragments where old and young trees were studied, genetic diversity is lower and inbreeding is higher in more recently established trees. Levels of genetic diversity in old trees from forest fragments are comparable with young samples from continuous forest, suggesting that the decline in diversity is unlikely to be a simple generational effect (Table 1). This exploratory comparison of young and old trees therefore provides no evidence for any post-bottleneck genetic recovery, although greater sampling from both forest fragments and continuous forest is required for robust statistical comparison of these measures between age groups.

In this investigation, samples were drawn from forest fragments that were larger than those reported in other temperate forest fragmentation studies. These *F. sylvatica* samples were distributed over a similar or smaller spatial scale (6, 9–12). Thus, the greater fragmentation effects that we report here are not a consequence of smaller fragment sizes or larger interpopulation distances when compared with previous studies. Given that beech is a highly outcrossing species, the level of inbreeding detected in the forest fragments (0.127) is surprisingly high, particularly because no significant inbreeding was detected in the continuous forest samples (Table 1) [theoretical values of F_{is} range from -1.0 (all individuals heterozygous) to $+1.0$ (no observed heterozygotes)]. Theoretical values of F_{st} vary from 0 (undifferentiated populations) to 1 (completely differentiated populations), although this theoretical maximum is unlikely to be reached in natural populations (22). In a study of sessile oak (*Quercus petraea*) populations in Ireland (11), F_{st} was found to vary between 0.001 and 0.024, with higher values up to 0.036 found only when comparing Irish populations with those in France and Spain. In common with European beech, sessile oak is a highly outcrossing, wind-pollinated species (11). We report F_{st} of 0.029 between beech forest fragments. This value of F_{st} is low in relation to the theoretical maximum; however, it is relatively large when considering the spatial scale of study and the mating characteristics of the species. Given that F_{st} between our continuous forest samples is 0.010, it is remarkable that the fragmentation of beech along the single mountain ridge we studied ($\approx$10 km long) has led to greater differentiation between populations than was detected for sessile oak populations throughout Ireland.

Despite the view that wind-pollinated trees may be at lower risk from habitat fragmentation, there is evidence that wind-dispersed pollen can be limiting (30). The population genetic effects of habitat fragmentation that we report here are corroborated by ecological work on beech (31) and blue oak (*Quercus douglasii*) (32) and genetic study of pollen movement in California valley oak (*Quercus lobata*) (33). These studies demonstrate reduced pollen availability to wind-pollinated trees growing in thinned and fragmented stands, where reduced density and increased isolation leads to a reduction in pollen donors (30, 32, 33). In beech and blue oak, the reduction in pollen donors results in reduced reproduction, as a predicted consequence of decreased availability of compatible pollen and elevated levels of inbreeding (31, 32).

Previously there has been little support for theoretical predictions that forest fragments should be more inbred, more differentiated, and lower in diversity than their continuous forest counterparts (3). In a current review of work on 23 neotropical trees (4), limited effects on adult and juvenile trees were found in only five of the fragmentation studies assessed, and none supported these predictions in their entirety. Temperate tree species repeat this pattern (5, 6, 9–12). One of the difficulties in assessing the effects of habitat fragmentation on tropical trees is that in many cases it results from deforestation that is recent enough to have occurred within the lifetime of surviving trees (4, 13), a scenario that is repeated in some temperate studies (6, 12). Despite this difficulty, studies on tree seedlings established after forest fragmentation have shown altered patterns of gene flow into remnant populations of both tropical and temperate species (4, 6, 12, 15, 16). These studies demonstrate that fragmentation is likely to affect the genetic structure of future adult populations. However, because of the smaller age range of seedlings in any sample, they result from far fewer mating events than a

sample of adult trees (16), and few will survive to adulthood. Therefore, study over a much longer time scale is necessary for the genetic effects of forest fragmentation to become clear (4).

Historic temperate deforestation is often so extensive that fragments and continuous forest no longer coexist (9–11). This complicates assessment of the effects of fragmentation because geographical gradients in genetic diversity and population genetic structure can be generated during species migrations (5, 17, 18), and their direction may contradict theoretical predictions (17). Consequently, comparison of forest fragments with continuous forest in other areas of a species' range may not provide a reliable estimate of prefragmentation levels of genetic diversity and population genetic structure. This lack of comparative pre- and postfragmentation data limits the information that can be gained from remnant populations and may partly explain why only limited effects of forest fragmentation have been reported previously. However, unexpectedly high levels of gene flow and genetic diversity have been reported for very small remnant populations of some temperate trees [a minimum of 4–10 individuals or 0.4- to 1.4-ha area (6, 9, 10, 12)], demonstrating the need for further study of the maintenance of genetic diversity in remnant forest populations.

In conclusion, our work shows that fragmentation of beech forest has resulted in genetic bottlenecks and the breakup of panmictic breeding in this species. This has led to increased inbreeding, population divergence, and the loss of genetic diversity from remaining populations. This study provides support for the predicted effects of habitat fragmentation drawn from population genetic theory. Where population structure and breeding systems are disrupted by forest fragmentation as we demonstrate here, elevated inbreeding within remnants may be both cause and consequence of further decline of fragmented tree populations (2, 32). The increase in genetic isolation of these fragmented populations, acting in concert with the reduction of their genetic diversity, is likely to have a negative impact on their persistence and evolutionary potential in the face of environmental change (1, 7, 8). These risks must be taken into account in the conservation and management of remaining natural and seminatural forest throughout the world.

Methods

Study Species and Site. The European beech (*F. sylvatica* L.) is a monoecious, diploid, late-successional tree that dominates temperate forests over ≈17 million ha of Europe. It is highly outcrossing and largely self-incompatible with irregular synchronous flowering (masting) events (17, 31). Beech colonized the Montseny Mountains of Catalonia (northeast Spain) >4,000 years ago (17). It now forms extensive forest in the temperate zone, typically >1,000 m above sea level. The heavily forested northeast ridge of these mountains rises to 1,712 m above sea level and includes 2,830 ha of near-continuous beech forest. The southwest ridge rises to 1,344 m above sea level, its summit formed by a central plain that was largely deforested by the 15th century (28, 29). On this lower ridge, beech is now restricted to a series of small forest fragments surrounding the pastures of the central plain (Fig. 2). Individual fragments cover an area of up to 52 ha and are estimated to be at least 600 years old.

Sampling and Sample Preparation. Forest fragments and continuous forest samples were taken from mature, closed-canopy *F. sylvatica* forest. Neighboring forest fragment samples were separated by between 0.71 and 2.08 km. The maximum distance between forest fragment samples was 5.82 km. For continuous forest, neighboring samples were separated by between 0.86 and 2.08 km with a maximum distance between samples of 8.17 km. Sampled fragments ranged between 21 and 52 ha in area. Beech population density in the continuous forest ranges from 785 to 1,471 trees per ha (based on records from the 2000–2001 Spanish National Forest Inventory IF3). We did not observe a consistent difference in density between the forest fragments and continuous forest.

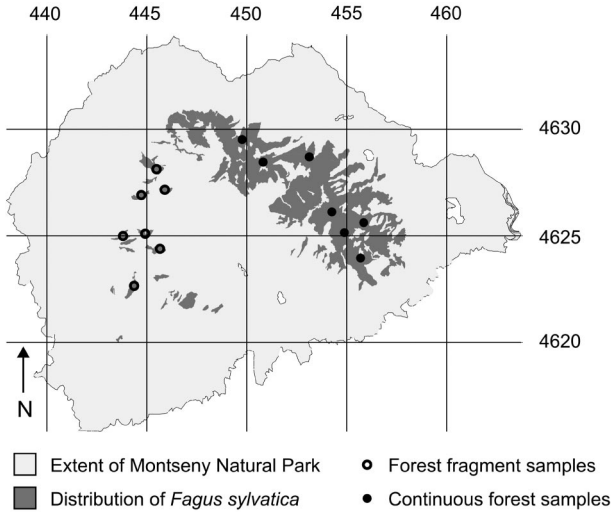

Fig. 2. Location of *F. sylvatica* samples taken within the Montseny Natural Park, Catalonia, northeast Spain. Grid lines are marked with Universal Transverse Mercator coordinates in kilometers.

We sampled newly expanding leaves or leaf buds from 50 young trees (>2 m in height) distributed randomly in each of fourteen 20-ha plots, seven in forest fragments and seven in continuous forest (Fig. 2), during spring 2004. In two of the forest fragments, additional leaf samples were taken from 50 mature trees, selected as the oldest neighboring tree to each young tree sampled. Leaves were dried immediately in fine-grain silica gel. Leaf samples were prepared for PCR according to the combined methods of Jump *et al.* (34) and Xin *et al.* (35). Briefly, 0.5 cm^2 of dried tissue was ground for 30 s in 100 μl of a buffer comprising 100 mM NaOH and 2% Tween 20. The mixture was spun at 3,220 relative centrifugal force for 5 min, and 30 μl of the supernatant was mixed with 470 μl of a buffer comprising 100 mM Tris·HCl (pH 8) and 2 mM EDTA. One microliter of this extract was used directly in each PCR.

Genotyping. Individuals were genotyped at six highly variable microsatellite loci originally isolated in *F. sylvatica* (FS1-03, FS1-15, FS3-04, and FS4-46) (36), *Fagus crenata* (FCM5) (37), and *Castanea sativa* (CsCAT14) (38). $MgCl_2$ concentrations and cycling conditions were as reported in the original publications. PCR was performed in 15 μl of buffer comprising 50 mM KCl; 10 mM Tris·HCl (pH 8.3); 0.001% gelatin; 200 μM each dATP, dCTP, dGTP, and dTTP; 1% poly(vinylpyrollidone)-40; 0.125% BSA(V); 5 pmol each of forward and reverse primer; and 0.25 units (approximate) of DNA polymerase (Institut de Recerca i Tecnologia Agroalimentàries, Cabrils, Barcelona; isolated according to a standard protocol). Forward primers carried a fluorescent label (Applied Biosystems). Each set of 96 PCRs included a negative (water) and positive (known genotype) control. Alleles belonging to the six different loci were segregated on a 3100-Avant genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Fragment sizes were determined with reference to a Liz-500 size standard (Applied Biosystems) for each sample injection by using the program GENESCAN (Version 3.7).

Statistical Description and Analysis. Observed and expected heterozygosities, allele frequencies, and Nei's (23) genetic distance were calculated by using Version 6 of the program GENALEX

(39). The difference in the number of rare alleles present in pooled continuous forest and pooled fragmented forest samples was tested by using a χ^2 contingency table test. Calculation of allelic richness, within-population gene diversity (H_S) (40), *F*-statistics [using the estimators of Weir and Cockerham (41)], deviation from HWE, population differentiation, and comparison between grouped fragmented forest and continuous forest samples was performed in FSTAT V.2.9.3.2 (a program for estimating and testing gene diversities and fixation indices; www2.unil.ch/popgen/softwares/fstat.htm, Version 2.9.3). The 95% confidence intervals for F_{is} and F_{st} (bootstrapped over loci) and standard errors (jack-knifed over loci) were calculated in the same program. Units of randomization and justification of Bonferroni corrections are described in FSTAT. Standard error of allelic richness, H_O and H_S, was calculated from the seven population values in each group.

The program BOTTLENECK V.1.2.02 (21) was used to test for recent reduction in effective population size for each sample. This program was run under a two-phase model of evolution that generally fits microsatellite evolution better than either pure stepwise or infinite allele models (42). Ten thousand simulations were performed for each sample based on a two-phase model consisting of 90% single-step mutations and 10% multistep changes. A Wilcoxon sign-rank test was used across loci to test for observed gene diversity excess when compared with gene diversity predicted at equilibrium on the basis of the observed number of alleles (43). A one-tailed Fisher exact test was used to test the hypothesis that bottlenecked populations occur predominantly in forest fragments.

The relationship between Nei's (23) genetic distance and the corresponding linear geographic distance between all possible pairwise population comparisons within each group was assessed by using a Mantel test and reduced major axis regression. These tests were performed by using IBDWS (24).

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