Genetic Structure of *Rhododendron ferrugineum* at a Wide Range of Spatial Scales

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Abstract

*Rhododendron ferrugineum* L. (Ericaceae) is a subalpine shrub found throughout the Pyrenees and Alps at elevations of 1600–2200 m. We examined relationships between genetic and geographic distance, using 115 dominant amplified fragment length polymorphism (AFLP) markers to assess genetic structure over a wide range of spatial scales. We sampled 17 sites with distances of 4 km to more than 1000 km between them. At these scales we detected no association between geographic distance and genetic distance between populations. This suggests that genetic drift and gene flow are not in equilibrium for these populations. This pattern could have resulted from recent and rapid postglacial colonization, from more recent human disturbance, or as a function of frequent and random “natural” long-distance colonization. At two of our sites we used transects (two horizontal and two vertical with respect to slope at each site) to sample at distances ranging from 10 m to more than 5000 m. At this scale we observed a positive relationship between genetic and spatial distance along two vertical transects, one at each site. We hypothesize that isolation-by-distance at this smaller scale is a function of restricted gene flow via seed dispersal.

In most organisms, movements of individuals are constrained relative to the entire range of the species. Thus the probability of gene flow decreases with spatial distance. This is the basis of Sewall Wright’s isolation-by-distance model, whereby proximal individuals tend to be more similar genetically than distant ones (Wright 1938, 1940). The model was originally developed for a continuously distributed population (Wright 1940), but was later extended to a stepping stone model with discrete populations (Kimura and Weiss 1964; Malécot 1955; Wright 1943). However, more recently it has been shown that the relationship between spatial and genetic distance is more complex than a simple function of the probability of gene flow.

One underlying assumption of most isolation-by-distance models is that populations at the scale considered have attained an equilibrium between genetic drift (which leads to population differentiation) and gene flow (which leads to genetic homogenization) (McCauley 1993; Whitlock and McCauley 1999). Thus, when disequilibrium occurs, measures of population differentiation alone are likely to provide misleading information about gene flow (Whitlock and McCauley 1999). However, by examining the relationship between spatial distance and genetic distance it may be possible to infer the relative effects of gene flow and drift on population structure, and thereby describe possible historical scenarios for groups of populations or geographic areas (Hutchison and Templeton 1999; Roussel 1997; Slatkin 1993). Thus, with drift-gene flow equilibrium, genetic distance should increase monotonically with spatial distance. If there is no equilibrium then a relationship will not be evident and the relative effect of gene flow will determine the variance in genetic distance values. A stronger effect from genetic drift will result in a wide variance in genetic distance values across all spatial scales, whereas high rates of gene flow will constrain genetic distance to low values and therefore a lower variance.

A third alternative is lack of equilibrium, but with gene flow more effective at smaller spatial scales and drift more effective at wider scales. Several factors can lead to a lack of equilibrium between genetic drift and gene flow, including recent colonization, rapid range expansion, and range fragmentation. Intermediate levels of equilibrium are also possible. Hutchison and Templeton (1999) discuss the various effects of the above scenarios and how they may...
influence the relationship between spatial and genetic distance. Here we use this approach to examine the genetic structure of the shrub *Rhododendron ferrugineum* across its range in Europe.

Species ranges in temperate regions have changed drastically over the last 20,000 years with warming after the last glacial maximum and subsequent climate oscillations (Wright 1993). Although range changes will depend on the ecology, adaptability, and dispersal ability of each species in question, some general patterns can be inferred, providing a useful framework for biological and geological studies (Bermingham and Moritz 1998; Hewitt 2001; Huntley and Prentice 1993; Taberlet et al. 1998). Species that live above the tree line have the potential to survive in glaciated areas and may become isolated on ice-free mountains above glaciers (nunataks), a scenario inferred for some alpine plants (Stehlik et al. 2001). Species restricted to lower elevations or warmer habitats are forced to shift distribution during climate change. In North America, where most mountain ranges run north to south, many species were pushed south during glaciation and were able to reinvade northward during subsequent glacial retreat (Webb et al. 1993). In Europe, the situation was somewhat more complex because the Mediterranean Sea and Alps acted as barriers to southern range movement. This may have resulted in extinction of some species and refuge for others, often in multiple glacial refugia on the Iberian, Italian, and Balkan peninsulas (Taberlet et al. 1998).

A species’ dispersal ability would then affect subsequent range expansion out of the refugia. Admixture between gene pools from the different refugia would depend on the extent to which the Pyrenees and Alps were barriers to dispersal out of glacial refugia, as well as the level of subsequent gene flow, which can be different between maternally inherited and biparentally inherited markers. As phylogeographic and population genetic data are gathered from a variety of species, more can be learned about general patterns of range change since the Pleistocene (Hewitt 2001; Taberlet et al. 1998). We examined the population genetic structure of a subalpine shrub over a wide range of spatial scales in an attempt to infer historical patterns from current population genetic structure.

*Rhododendron ferrugineum* L. (Ericaceae) is distributed widely in the Alps and Pyrenees, where it can dominate communities between 1600 and 2200 m, especially in areas where grazing pressure has subsided (Escaravage et al. 1998). The species is self-compatible and reproduces through selfing and outcrossing (Escaravage et al. 1997) and through vegetative spread down slopes (Pornon et al. 1997). The latter can result in clones up to 20 m². The longest extent in one dimension that has been detected for a clone of *R. ferrugineum* (using genetic markers) is 6 m (Escaravage et al. 1998). Because *R. ferrugineum* is a successional species (occurring temporally between forest and pasture), it is possible that an equilibrium has not been reached between genetic drift and gene flow among most populations, but we do not know if this would be evident at all spatial scales. Furthermore, range expansion during glacial retreat has likely influenced the relationship between spatial distance and genetic distance. We examined these issues using *R. ferrugineum* populations sampled from across the range to examine genetic structure from a scale of tens of meters to more than 1000 km. The objectives of the study were to examine the relationship between spatial and genetic distance at different geographic scales, to infer the scale at which equilibrium occurs, and to compare population structure at different angles to the slope of a mountain.

### Materials and Methods

#### Sampling

We sampled 10 plants from each of 17 sites, 2 in the Pyrenees and 15 in the Alps (Table 1, Figure 1), covering most of the range of the species. Distances between these sites ranged from 4 km to 1028 km. Leaf tissue was sampled at 10 m intervals to ensure that adjacent samples were unlikely to be part of the same clone. At each of two sites (site 8, Grand Sure; site 12, Côte des Salières) we sampled at a finer spatial scale by taking two parallel transects across the slopes (“horizontal” transects) and two parallel transects up and down the slopes (“vertical” transects), for a total of eight transects. Parallel transects were approximately 100 m apart and we sampled along each transect at distances ranging from 10 m (closest pairs) to 5070 m (total transect length) for horizontal transects and 10 m to 794 m for vertical transects. We sampled 18–23 plants per transect. Leaf tissue was placed in vials containing silica gel and stored at room temperature.

#### Laboratory Protocols

DNA was extracted from 20 mg of dried leaf tissue using the DNaseasy Plant Mini kit from Qiagen, following the manufacturer’s protocols. DNA concentration was estimated by fluorimetry with the PicoGreen ds DNA Quantification kit (Molecular Probes Inc.).

### Table 1. Location and elevation of sites where *R. ferrugineum* was sampled

<table>
<thead>
<tr>
<th>Site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Elevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innsbrück</td>
<td>47°07′ N</td>
<td>11°40′ E</td>
<td>1800 m</td>
</tr>
<tr>
<td>St. Gothard</td>
<td>46°33′ N</td>
<td>8°34′ E</td>
<td>2100 m</td>
</tr>
<tr>
<td>OberPass</td>
<td>46°39′ N</td>
<td>8°40′ E</td>
<td>2000 m</td>
</tr>
<tr>
<td>SplugPass</td>
<td>46°30′ N</td>
<td>9°20′ E</td>
<td>2000 m</td>
</tr>
<tr>
<td>Chablais</td>
<td>46°13′ N</td>
<td>6°30′ E</td>
<td>1700 m</td>
</tr>
<tr>
<td>Crêt Neige</td>
<td>46°14′ N</td>
<td>5°55′ E</td>
<td>1500 m</td>
</tr>
<tr>
<td>Vercors</td>
<td>45°04′ N</td>
<td>5°36′ E</td>
<td>1600 m</td>
</tr>
<tr>
<td>Grand Sure-Chartreuse</td>
<td>45°20′ N</td>
<td>5°43′ E</td>
<td>1650 m</td>
</tr>
<tr>
<td>Collet d’Allevard</td>
<td>45°23′ N</td>
<td>6°08′ E</td>
<td>1600 m</td>
</tr>
<tr>
<td>St. Agnes</td>
<td>45°13′ N</td>
<td>6°00′ E</td>
<td>1650 m</td>
</tr>
<tr>
<td>Chamrouselle</td>
<td>45°07′ N</td>
<td>5°54′ E</td>
<td>1760 m</td>
</tr>
<tr>
<td>Côte des Salières</td>
<td>45°02′ N</td>
<td>5°53′ E</td>
<td>1900 m</td>
</tr>
<tr>
<td>Alpe d’Huez</td>
<td>45°07′ N</td>
<td>6°05′ E</td>
<td>1950 m</td>
</tr>
<tr>
<td>Galibier</td>
<td>45°03′ N</td>
<td>6°23′ E</td>
<td>2300 m</td>
</tr>
<tr>
<td>Queyras</td>
<td>44°50′ N</td>
<td>6°49′ E</td>
<td>2100 m</td>
</tr>
<tr>
<td>Canigou (Pyrenees)</td>
<td>42°32′ N</td>
<td>2°30′ E</td>
<td>2000 m</td>
</tr>
<tr>
<td>Néouvielle (Pyrenees)</td>
<td>42°56′ N</td>
<td>0°07′ E</td>
<td>2000 m</td>
</tr>
</tbody>
</table>
Amplified fragment length polymorphism (AFLP) protocols followed Vos et al. (1995): 20–40 ng of DNA was digested and ligated in an 11 µl reaction containing 1× T4 ligase buffer, 50 mM NaCl, 0.55 mg BSA, 1 U T4 ligase (Roche Molecular Biochemicals), 1 U MseI, 5 U EcoRI, and 0.9 µM of each of the two adapters. The mixture was incubated in a tube for 2 h at 37°C then diluted 10-fold.

Preselective polymerase chain reaction (PCR) used 3 µl of the diluted digestion-ligation mixture in a total volume of 12.5 µl containing 1× Taq buffer, 1.5 mM MgCl₂, 0.12 mM each dNTP, 0.2 µM EcoRI primer (GACTGCGTAC-CAATTC), 0.2 µM Mse primer (GATGAGTCCTGAGTAA-CAC), and 0.25 U AmpliTaq polymerase (Applied Biosystems). Reactions were held at 95°C for 10 min followed by 13 cycles of 94°C (30 s), 65°C → 56°C (1 min), and 72°C (1 min) (annealing temperature was reduced 0.7°C on each cycle), followed by 23 cycles of 94°C (30 s), 56°C (1 min), and 72°C (1 min), with a final 10 min extension at 72°C. The selective PCR was repeated on all samples with a second MseI primer (GATGAGTCCTGAGTAA-CAC).

We selected 15 DNA samples at random that were each run twice to test repeatability. Samples were run with a fragment size marker (GeneScan 500 ROX, Applied Biosystems) on an ABI 377 and AFLP bands were visualized with GeneScan software. Bands with less than 10% of the maximum intensity for that band on the same gel were treated as absent from the individual. Bands at two positions on the gels were inconsistent among repeated samples of the same plant. Therefore data from these band positions were excluded from all individuals. For the repeatable AFLP bands, all individuals were coded as band presence or absence. We scored only bands in the size range of 50–500 bp.

Figure 1. Map of western Europe showing sampling sites of *R. ferrugineum*.
Analyses

Transects

We first examined the relationship between genetic similarity and spatial distance between pairs of individuals within transects (the smallest scale in this study). We calculated similarity coefficients following the method of Nei and Li (1979), which is based on that of Dice (1945). This approach ignores matching absences between individuals, which can be difficult to interpret in PCR-based markers where several factors can result in amplification failure. Dice similarity values were calculated using NT SYS (Rohlf 1997) for all pairs of individuals and then converted to distance (1 – similarity). For each of the eight transects, pairwise matrices of Dice distance and physical distance (from transect measurements) were compared with a Mantel test (Mantel 1967) using NT SYS, employing 1000 randomizations.

Populations

We first examined the genetic similarity among individuals across populations with principle component analysis using PROC FACTOR in SAS (SAS Institute Inc. 1999). Genetic relationships among the 17 sampled populations of *R. ferrugineum* were estimated in several ways. For all pairs of populations we used the software TFP GA (Miller 1997b) to calculate the genetic distance (D) of Nei (1978) and the $F_{ST}$ estimator $\theta$ of Reynolds et al. (1983). Because AFLP markers are dominant, our analyses of D and $\theta$ assumed that samples were in Hardy-Weinberg proportions at each site. Based on this assumption, allele frequencies were estimated by the Taylor expansion estimate (Lynch and Milligan 1994). We also used analysis of molecular variance (AMOVA) (Excoffier et al. 1992) to calculate pairwise estimates of $\phi_{ST}$ (an $F_{ST}$ analog) based on marker phenotype variation, making no explicit assumptions about genetic control of the markers. We used AMOVA-PREP (Miller 1997a) to prepare input files for analysis using WinAMOVA (Excoffier 1992). Pairwise estimates of genetic differentiation were then compared by correlation coefficients and plotted against the geographical distance between population pairs. For each of the three measures of genetic distance, pairwise matrices of genetic distance and geographic distance were compared with a Mantel test (Mantel 1967) using TFP GA, employing 1000 randomizations.

To compare our results at the larger spatial scales (5–1000+ km) to those of the smaller scale of transects (10–5000+ m), we recalculated Dice distance values by selecting one individual at random from each of the 17 sites, performing a Mantel test as above. In addition to these estimates of genetic differentiation between pairs of populations, we also estimated the overall $F_{ST}$ and $\phi_{ST}$ among all 17 populations. For $F_{ST}$, we calculated $\theta$ by TFP GA, and used 1000 bootstrap replicates (over loci) to estimate 95% confidence intervals. For $\phi_{ST}$ we used WinAMOVA (Excoffier 1992), which uses a randomization procedure (random allocation of individuals and genotypes to populations) to obtain a $P$ value.

Results

We scored a total of 115 polymorphic AFLP markers for the two primer pairs. Associations between genetic and spatial distance were evident at the smaller spatial scales (within transects). Among the eight transects, two showed significant positive, though weak, associations between genetic and spatial distance (Figure 2). Both significant associations were for vertical transects, one at each site. We detected no two adjacent samples on a transect with identical AFLP profiles, indicating that we successfully avoided resampling the same clone.

We observed few clear geographic patterns at individual loci. Frequencies varied between populations, but we found no AFLP band fixed in any population or in any geographic region. The principle component analysis found little predictive correlation among loci. The first principle component accounted for 11.3% of the variance, the second for 8.5%, and the third for 5.5%. A plot of individuals for the first two principle components (Figure 3) revealed little clustering of plants from each population. Although individuals from some populations (12, 16, and 6) tended to cluster in the same region of the plot, there was nevertheless considerable overlap among populations. The average $\theta$ among the 17 populations was 0.23 (SD = 0.014; 95% confidence interval [CI] 0.20–0.25), the average Nei’s D was 0.094 (SD = 0.037), and the overall $\phi_{ST}$ was 0.205 ($P < .001$).

There appears to be little correlation between genetic and geographic distance based on plots of population pairs (Figure 4). $\phi_{ST}$ and $\theta$ ranged from 0.07 (populations at 16 km apart) to 0.45 (populations at 496 km apart). We first compared the genetic distance measures, disregarding geographic distance. The matrices for all three measures of genetic differentiation were highly correlated with one another across population pairs ($r = 0.94$ for $D$ versus $\theta$; $r = 0.75$ for $D$ versus $\phi_{ST}$; $r = 0.86$ for $\phi_{ST}$ versus $\theta$). These correlations between genetic and phenotypic estimates of genetic distance suggest that the effect of assuming dominance for $\theta$ and $D$ imposes little or no effective bias. Mantel tests revealed little association between the matrices of genetic versus geographic distance: for Nei’s $D$, $r = 0.12$ ($P = .55$); for $\theta$, $r = 0.043$ ($P = .60$); and for $\phi_{ST}$, $r = 0.15$ ($P = .22$). Removing the Pyrenean populations from the analysis also revealed no detectable association (data not shown). Based on these results we can reject the hypothesis of a gene flow–genetic drift equilibrium at the continental scale (Hutchison and Templeton 1999). When we resampled one individual at random from each population, the mean correlation coefficient between Dice and geographic distance across 1000 replicates was $r = 0.08$ (SD = 0.12) (Figure 5). The wide distribution of correlation coefficients (Figure 5) further indicates the negligible association between geographic and genetic distance. Thus all approaches indicate a weak relationship between these variables at the larger scale of 5–1000+ km.

Discussion

Continental-Scale Patterns

Sampled populations of *R. ferrugineum* varied genetically based on our AFLP markers; measures of population
Figure 2. Relationship between spatial distance and Dice genetic distance at each of eight transects of *R. ferrugineum*. A and B are vertical transects, C and D are horizontal relative to the slope of the mountainside.
differentiation ($D$, $\theta$, and $\phi_{ST}$) are neither unusually low nor high compared to other published studies and reviews based on various polymorphic markers (Gottlieb 1981; Hamrick and Godt 1989; Sawkins et al. 2001). No population nor region was characterized by a fixed allelic difference. Instead, all variation detected was in the form of AFLP frequency differences. Moreover, we detected no association between genetic distance and geographic distance for population pairs at the continental scale. Based on the scatter plots, the results are most consistent with Hutchison and Templeton’s (1999) scenario that depicts a lack of regional equilibrium, with genetic drift perhaps slightly more influential than gene flow at the regional scale (hundreds of kilometers). If genetic drift were appreciably more influential than gene flow, we would expect to see a wider spread of $\theta$ values (Hutchison and Templeton 1999), including population pairs that had diverged more than those we observed. Several unrelated phenomena can result in a lack of equilibrium. Causative evolutionary processes include the possibility of recent and rapid range changes. These could be associated with climate change at different time scales, as well as the more recent colonizations associated with human disturbance and movement, or via other agents of dispersal.

The climate of the northern hemisphere has oscillated throughout the Holocene (Huntley and Prentice 1993). Initial warming after the last major glaciation was probably rapid. Cold phases have occurred since then at approximately 59,000 years ago, 26,000 years ago, 13,000 years ago, 11,500 years ago, 8200 years ago, and more recently at 700–200 years ago (Huntley and Prentice 1993). The onset and duration of these events probably varied, as did the interacting effects on precipitation levels. Nevertheless, community structure, species ranges, and population distributions have been in constant flux (Wright 1993).

How these events have influenced the genetic history of *R. ferrugineum* is unclear. There are pollen records of *R. ferrugineum* from 9300 years ago from the central Alps region,
close to population 4 of our study (Wick 1994), so it is likely that the species has been present throughout the Alps for at least that long. However, it is unlikely that the range of the species has remained constant. The fluctuations in climate and concomitant vegetation shifts are likely to have resulted in an effect of genetic drift. Our scatter plots are consistent with this, but our genetic distance values indicate a lack of population-specific allelic fixation across the entire range of the species. This is consistent with a secondary influence of gene flow (but uncorrelated with distance). Such apparently random gene flow events could be natural (not human induced) and combined with local colonization events.

Alternatively, the patterns could be attributed to human-induced disturbance, including the movement of material during migration as well as via livestock. A secondary effect from human disturbance is the recent cessation of grazing, which has increased the aerial extent of *R. ferrugineum*. However, this has probably not been responsible for rapid colonization at the regional or continental levels. Pornon and Escaravage (1999) showed that populations in the French Alps that consist of pure stands of *R. ferrugineum* are the result of clonal spread and that most of the seed recruitment (i.e., genetic colonization) occurred prior to expansion of *R. ferrugineum* into these “closed” communities. Thus regional colonization is more likely a function of random seed dispersal rather than the cessation of grazing.

**Fine-Scale Genetic Structure**

Since we detected a positive relationship between spatial and genetic distance on only two (of four) vertical transects, we are unable to reject the hypothesis of equilibrium at the smaller spatial scale. Although the pattern was weak, it is consistent with what we know of the biology of *R. ferrugineum*. Individuals can spread genes via three processes. First, at small spatial scales, colonization can be manifested through clonal spread. In the current study we sampled at distances beyond which clones have been detected previously, and we did not detect multiple sampling of the same clone. Thus we think that clonal spread is not responsible for the patterns we observed. Second, seeds are dispersed by gravity, and it is possible that spring runoff could lead to more dispersal down slope, resulting in an isolation-by-distance pattern in this dimension. Third, alleles can be spread via pollen on visiting insects, mostly *Apis* and *Bombus*. Although strong unidirectional foraging has been observed in *Bombus* (Osborne et al. 1999), it is not known if this is more likely to occur down slope relative to across slope. If there are such patterns, they may vary among sites and depend on hive location. Furthermore, it is also not clear if such pollinator behavior could lead to an isolation-by-distance pattern (Waser 1993). More direct experiments would be needed to examine the effects of seed dispersal and test the directionality of pollinator movements.

**Conclusion**

We detected no obvious relationship between genetic and spatial distance at large spatial scales (4–1000 km) in *R. ferrugineum*. Conversely, the detection of such a correlation along local transects suggests the possibility of an equilibrium between gene flow and genetic drift below the scale of 2 km. However, this pattern was relatively weak and was detected on vertical transects and not horizontal transects along a mountainside.

Inferring the causative relationships of historical climate with distribution and current genetic structure is one of the most challenging, yet productive fields in evolutionary biology. Only by obtaining data on a large sample of different species can we begin to make predictive hypotheses about how these factors are related. The most rewarding studies are likely to come from species for which we have good historical distribution data, usually in the form of pollen deposition maps. But it may also be possible to examine species for which more recent historical data are available. However, inferring equilibrium effects from genetic structure is very dependent on the type of markers used. Different rates of allele accumulation will affect the time scale at which equilibria can be detected. This is analogous to the variation in estimates of effective population size using heterozygosity values from different markers (Hedrick 1996). Thus future studies of *R. ferrugineum* will include analyses of chloroplast DNA haplotypes as well as more rapidly evolving markers such as microsatellites.

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