

Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (Ericaceae) populations

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Abstract

Two populations of *Rhododendron ferrugineum* growing at subalpine level in the Pyrenees (France) were studied in two sites (Bethmale and Mourtis). Identification and delimitation of genets were inferred from amplified fragment length polymorphism (AFLP) markers, along a closure gradient (from meadow to more closed heath) in each site. Surface and age of genets, genotypic diversity (Simpson's index D), 'proportion distinguishable' genotypes and genetic relationships between genets were then estimated. Amplification of the 312 DNA samples with three selective primer pairs gave a mean of 98 detectable peaks (i.e. bands) per sample, with size ranging from 60 to 300 bp. In total 60% (Bethmale) and 70% (Mourtis) of the peaks were polymorphic, and a total of 31 and 23 multilocus genotypes were identified, in Bethmale and Mourtis, respectively. We inferred that pioneer genotypes began arriving 110 years ago mainly over a 40-year period in the Mourtis meadow, and began about 130 years ago over a 100-year period in the Bethmale meadow. After this pioneer stage, populations extended vegetatively. Two different patterns of genotypic dynamics can be identified. At Bethmale, population closure could have led to a dramatic loss of genets and to the selection of highly genetically related genotypes. In contrast, at Mourtis, genotypic diversity and genet density did not change fundamentally along the closure gradient. However the range of genetic diversity diminished from the open to the closed situation, suggesting that thinning could have occurred in the past.

Keywords: AFLP markers, closure gradient, frequency-dependent selection, genotypic similarity, genotypic variation

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Introduction

Low recruitment rates from seeds have been reported to be common features in clonal populations even if seed production is abundant (Eriksson 1989; Schmid 1990; Grabherr 1997; Pornon *et al.* 1997). Proliferation of genetically identical individuals (ramets) in a population in which intraspecific competition occurs between both clones, and ramets within clones, potentially leads to few aggressive clones dominating the population (Soane & Watkinson 1979). This was thought to dramatically impoverish genotypic diversity in clonal populations. The long life of clonal plants (Bell & Bliss 1979; Garcia & Antor 1995;

Steinger *et al.* 1996; Escaravage *et al.* 1998) which occupy the most favourable sites for seedling development over a long time may aggravate this trend. This led authors to suggest that a high degree of asexual reproduction is often associated with genetic monomorphism (Williams 1975; Harper 1977). However, Ellstrand & Roose (1987), Hamrick & Godt (1989) and Widén *et al.* (1994), in literature surveys of allozyme variation in clonal plants, recently concluded that clonal populations may have high genetic diversity. Such a high level of genotypic diversity has often been explained as a result of microsite heterogeneity that promotes the coexistence of clones through diversifying selection, as does frequency-dependent selection (Antonovics & Ellstrand 1984; Ellstrand & Roose 1987). Moreover, population maturation may lead biotic interactions between genets to change with time. For instance we can imagine that once self-thinning has

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eliminated failing clones, symmetrical competition (de Kroon *et al.* 1992) occurs among the surviving clones and allows them to coexist over a long time. Study of genotypic dynamics in *Solidago canadensis* has revealed such changes in clone interactions (Hartnett & Bazzaz 1985). If clones persist for a long time at a site, even very episodic recruitment may be sufficient to compensate for mortality and maintain a high level of genotypic diversity in the population.

When and how genets arrive at a site and how interactions between genets or ramets evolve with population maturation are key questions in the assessment of genotypic dynamics in clonal populations. Different patterns of seedling recruitment among plant species have led Eriksson (1989) and Jelinski & Cheliak (1992) to propose alternatives for population development which imply differences with regard to genetic diversity, genotypic structure, and ability to develop local adaptations in these populations: (i) seedling establishment occurs heavily during a short period early in population development, with no further seedling establishment after the initial colonizing phase (Initial Seedling Recruitment: ISR), in which case, loss of genet diversity may occur with time and decrease the capacity of a population to respond to changing environmental conditions; (ii) seedling recruitment occurs repeatedly (RSR) during population development; and (iii) seedling recruitment occurs in a 'window of opportunity' (RWO), episodically on a frequency scale of several decades or centuries depending on the perturbation. Unlike ISR, RSR and RWO may maintain genetic diversity at a high level in the population which is favourable to local adaptation. Although, during the last decades, the literature has become progressively enriched concerning genotypic structures in natural populations, the underlying processes related to its origin and its evolution have, until now, been poorly investigated and the ISR, RSR, and RWO models need much more investigation to be validated. The reason is that rare seedling recruitment, the long life of clones, diffuse clone boundaries, diffuse ramet replenishment into clones (Hutchings 1979; Pitelka 1984; Thompson *et al.* 1990), difficulties with estimating the age of clones, and differentiating ramets from genets, make it difficult or impossible to obtain reliable knowledge on population dynamics in these plants.

In this paper we consider the clonal ericaceous shrub *Rhododendron ferrugineum*. Recently Escaravage *et al.* (1998) investigated clonal diversity, using amplified fragment length polymorphism (AFLP) markers, in a dense population of *R. ferrugineum* with a closed cover for the last 50 years. They found a high level of genotypic diversity as they identified 32 genotypes on a 200-m² plot. The oldest clone was at least 260 years old. However, these findings did not provide a conclusive scheme of the origin and the

evolution of the genotypic structure during population development. More recently Pornon & Escaravage (1999) re-examined the data in the light of a previous study conducted on the same population (Pornon & Doche 1995a) and came to the plausible assumption that all or most clones became established during the early successional stages and that the current genotypic structure reflected the initial genotypic structure established long before the population closed. We suggested that the seedling recruitment pattern in *R. ferrugineum* was more consistent, albeit not entirely, with the ISR model. However, no conspicuous evidence of this was provided by the previous studies. Furthermore, it was impossible to determine whether self-thinning had occurred during population maturation. A question persisted about whether the smallest clones (about 1 m²) growing among the biggest ones (20 m²) were relics of previously more widespread or older clones rather than young clones. If confirmed, the former hypothesis would be more consistent with the ISR model. The latter hypothesis requires seedling recruitment to occur repeatedly and would be more consistent with the RSR or RWO models. However, because seed germination requires strong light (Bianco & Bulard 1974) seedling recruitment was assumed to be very unlikely in closed populations (Escaravage *et al.* 1998; Pornon & Escaravage 1999).

In this study we used a 'synchronic approach' (Lepart & Escarre 1983) to investigate the evolution of genotypic structure together with a closure gradient of *R. ferrugineum* heathlands extending downslope over meadows. In such a synchronic approach the closure gradient is hypothesized to account for a temporal colonizing gradient with different secondary successional stages. In our study, successional stages were meadow, open heathland and closed heathland. Meadow with isolated *R. ferrugineum* represents the pioneer stage of seedling arrival on the site and open heathland and closed heathland represent increasingly advanced stages in population dynamics. Although it is often difficult to be sure that the environmental context has not changed during population development, a synchronic approach has been abundantly used in studying vegetation dynamics and is often the sole means available to investigate populations with long-term dynamics such as in *R. ferrugineum*. The genotypic structure was inferred from AFLP markers. The main objectives of this study were to ask the following questions: (i) is there a particular population successional stage during which seedlings are recruited and the genotypic structure is primarily established?; (ii) does a successional stage exist during which seedling recruitment is prevented?; (iii) which model (ISR vs. RSR vs. RWO) is the *R. ferrugineum* population successional pattern consistent with?; and (iv) does population closure and maturation lead to dramatic changes in the genotypic structure?

Materials and methods

Study sites

The study was conducted just above the timberline at two mountain sites in the Pyrenees Centrales 150 km south of Toulouse (France). The first was Bethmale (42°51' N; 1°4' E) located 1600 m above sea level (a.s.l.) on a north-west facing slope of 35°. The other was situated at Mourtis (45°55' N; 0°47' E) 1700 m a.s.l. on a north-east facing slope of 19°. The average annual precipitation is approximately 1500–2000 mm at both sites. Usually snow cover persists at both sites until May/June. The geological substrate is gneiss-migmatite in Bethmale and a trias ophite-schist-sandstone mixture in Mourtis. On both sites *Rhododendron ferrugineum* grows on Typical Haplorthod to Umbric dystrochrept (Soil Survey Staff 1975) soils with a pH (H₂O) and C:N ratio, respectively, 4.2 and 17.2 at Bethmale and 3.9 and 20.5 at Mourtis.

Human use of the sites follows the general scheme observed in the Pyrenees (G. Jalut, personal communication). From the Bronze age, the forests progressively disappeared and the herbaceous species dominated subalpine vegetation from the Middle ages until now (Galop & Jalut 1994) as grazing pressure increased. On our study sites, livestock grazing has existed for millenia and has persisted until now. In 1884 at Bethmale and 1837 at Mourtis regulations were laid down to control grazing in mountain pastures and grazing remained relatively intensive until the end of the 1960s. Then it decreased as mountain depopulation occurred. Early in the eighties grazing pressure increased again and reached the current relatively high levels. So except for the 1970–80 period the environmental context has not basically changed over the centuries.

Species studied

Rhododendron ferrugineum L. (Ericaceae) is an evergreen shrub with well-branched trailing stems reaching a height of 70–80 cm. It has a large geographical range in the Alps and the Pyrenees (Ozenda 1985) where it often dominates in alpine communities between 1600 and 2200 m. It reproduces both sexually by selfed and out-crossed seeds (Escaravage *et al.* 1997) and vegetatively through layering (Pornon *et al.* 1997).

Experimental design

Sampling procedure. We carefully chose habitats homogeneous for slope, aspect, texture, and general appearance. These criteria determined the site and size of the plots. In Mourtis a rectangular plot of 16 × 6 m was selected parallel to the slope in the ecotone of a vast heathland extending

into a *Vaccinium myrtillus* stand. The *R. ferrugineum* population progressively increases in cover from scattered individuals in the lower part of the plot to dense and close vegetation in the upper part. In Bethmale we similarly selected a 17 × 7 m plot in the ecotone. However, because this plot did not include such a closed population as in Mourtis we selected a second more closed plot of 7 × 5.5 m, 15 metres upslope.

The position and limit of each *R. ferrugineum* genet (see below for methods of genet identification) was recorded on a map which was then computed (Mapinfo version 4.0, 1996) to determine the area and the population cover in each entire plot. Cover was then scored in three sub-plots at each site: meadow with isolated individuals (hereafter called meadow), heathland with up to 50% cover (open heathland), heathland with up to 80% cover (closed heathland). At Bethmale meadow and open heathland occurred within the 17 × 7 m plot while the 7 × 5.5 m plot accounted for the closed population. Because meadow was strikingly larger than both the open and closed heathlands, when needed, we sometimes pooled data (as mentioned below in the text) from heathlands in a single data set for statistical analysis.

At Bethmale, 'meadow' consisted of extensively grazed herbaceous vegetation while at Mourtis meadow was covered by a *V. myrtillus* heathland less frequented by livestock. As it was not possible to identify ramets, leaves of *R. ferrugineum* within each plot were collected at each intersection point of a 0.5 × 1 m grid. We chose a separation of 0.5 m parallel to the contour lines to reduce the risk of skipping nonlayering individuals because mature individuals are usually larger than 0.5 m. A separation of 1 m parallel to the slope was used because individuals usually spread down the slope. Leaves are immediately preserved in silica gel to prevent DNA degradation for the AFLP procedure. We analysed a total of 312 samples i.e. 186 in Bethmale and 126 in Mourtis.

DNA isolation and AFLP procedure. Forty milligrams of a dry leaf was extracted using the DNeasy™ Plant Mini Kit (QIAGEN) according to the manufacturer's handbook. After extraction, the DNA was quantified by fluometry and diluted in 20 µL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA; Sambrook *et al.* 1989).

The AFLP procedure is detailed in Escaravage *et al.* (1998). The three primer pairs used in selective amplification were: fluorescent (FAM) *EcoRI*-ACA/*MseI*-CTC, fluorescent (JOE) *EcoRI*-AAC/*MseI*-CAC, fluorescent (NED) *EcoRI*-AAG/*MseI*-CTG.

Estimation of genet age. It is impossible to know the true clone age by counting growth rings of ramets. However, Pornon *et al.* (1997) demonstrated the suitability of using the average annual length growth rate of aerial shoot to

estimate genet age by the fact that prostrate stems (genets extend downslope mainly by layering) and aerial stems usually have a similar annual elongation. Thus, the age of each clone was estimated using the average annual length growth rate of its aerial shoots and the area covered by the clone according Escaravage *et al.* (1998). Here the annual length growth rate of aerial shoots was measured for the last five years on 20 shoots of each nonlayering genet and on 50 shoots of each layering genet detected by AFLP. In order to estimate the average length of prostrate stems we also excavated seven (Bethmale) and nine (Mourtis) individuals from meadows and measured the length of all prostrate stems. The age of each genet was then estimated as follows: total length of the genet/average annual length growth of aerial shoots. Total length of the genet was the downslope length of the aerial part + the average prostrate stem length at the site.

Assessing 'sexual and asexual' components in population cover. In *R. ferrugineum* layering occurs when individuals are about 50–60 years old (Pornon *et al.* 1997). Until this age the genet corresponds to a seed-originated individual. Then the genet extends by producing ramets. A ramet is a member or modular unit of a clone, that may follow an independent existence if separated from the parent organism (Lincoln *et al.* 1998). Therefore, a clone patch can be divided into two different components: a 'sexual component', i.e. the cover of the seed-originated individual, and an 'asexual component', i.e. the cover of the layering-originated individuals (ramets). In this study we used the average cover of nonlayering individuals from meadows to estimate the sexual component of genets in all subplots. The asexual component can then be estimated from the total genet cover minus the sexual component. In this way, population cover built from sexual vs. asexual reproduction can be estimated over the entire plot.

Data analysis

The data were analysed using GENESCAN analysis software according to the user's manual (Perkin Elmer Corporation 1995). Each sample was scored for the presence or absence of peaks (i.e. bands) over a readable range of fragments (75–300 bp). Similarity in AFLP profiles between all possible pairwise of genets was expressed by the similarity index (Lynch 1988, 1990):

$$S_{xy} = 2n_{xy} / (n_x + n_y)$$

where n_x and n_y refer to the number of AFLP peaks in genets x and y , respectively, and n_{xy} was the number of bands shared by genets x and y .

Only polymorphic peaks were considered in the index calculation. The differences in similarity index between sites and between subplots within a site were statistically tested. Because the data failed to fit a normal distribution and because subplots had very different numbers of values, intersite variations were tested by the Wilcoxon–Mann–Whitney tests (SYSTAT 7.0.1, 1997) and intersubplot variations by Kruskal–Wallis tests. When Kruskal–Wallis tests detected a heterogeneity in data sets, we performed mean multiple comparison tests with a Student–Newman–Keuls test adapted to uneven data sets (Scherrer 1984) to highlight the deviant information.

Two measures of genotypic diversity were calculated following Ellstrand & Roose (1987): (i) the 'proportion distinguishable' was calculated as the number of genets detected (G) divided by the number of samples (n). Usually n represents the number of ramets sampled. However, because it was no longer possible to know this number in our study we did not consider the unit to be a ramet but a branch; and (ii) the complement of the Simpson index corrected for finite samples, D (Pielou 1969; Peet 1974):

$$D = 1 - [\sum_i (n_i - 1)] / n(n - 1)$$

where n_i is the number of samples of genets i and n is the total number of samples. D ranges from zero in a population composed of a single genet to one in a population where every individual sampled has a different genotype.

Results

On average *Rhododendron* cover was 56% at Mourtis and 48% at Bethmale. Cover in subplots of Mourtis reached 20% in meadow, 75% in open heathland and 98% in closed heathland, respectively. The values were 15% in meadow, 66% in open heathland and 97% in closed heathland in Bethmale.

Amplification of the 312 DNA samples with the three selective primer pairs gave a mean of 98 detectable peaks (i.e. bands) per sample, with sizes ranging from 60 to 300 bp. To avoid artefacts and ambiguities, only peaks with a high intensity were considered. In the Bethmale plot 60% of the peaks were polymorphic and allowed 31 multilocus genotypes to be identified; in Mourtis, 70% were polymorphic and indicated 23 multilocus genotypes. The spatial distribution of the genotypes on the plots is shown in Figs 1 and 2 for Bethmale and Mourtis, respectively. The limits of genets in both open and closed heathlands were often blurred and intermingling occurred. Parallel to an increase in average genet size, genet density (Genets. m⁻²) dramatically decreased from meadow to closed heathland at Bethmale (Table 1). In contrast at Mourtis, the increase in genet size was not paralleled by a reduction of genet density. The average

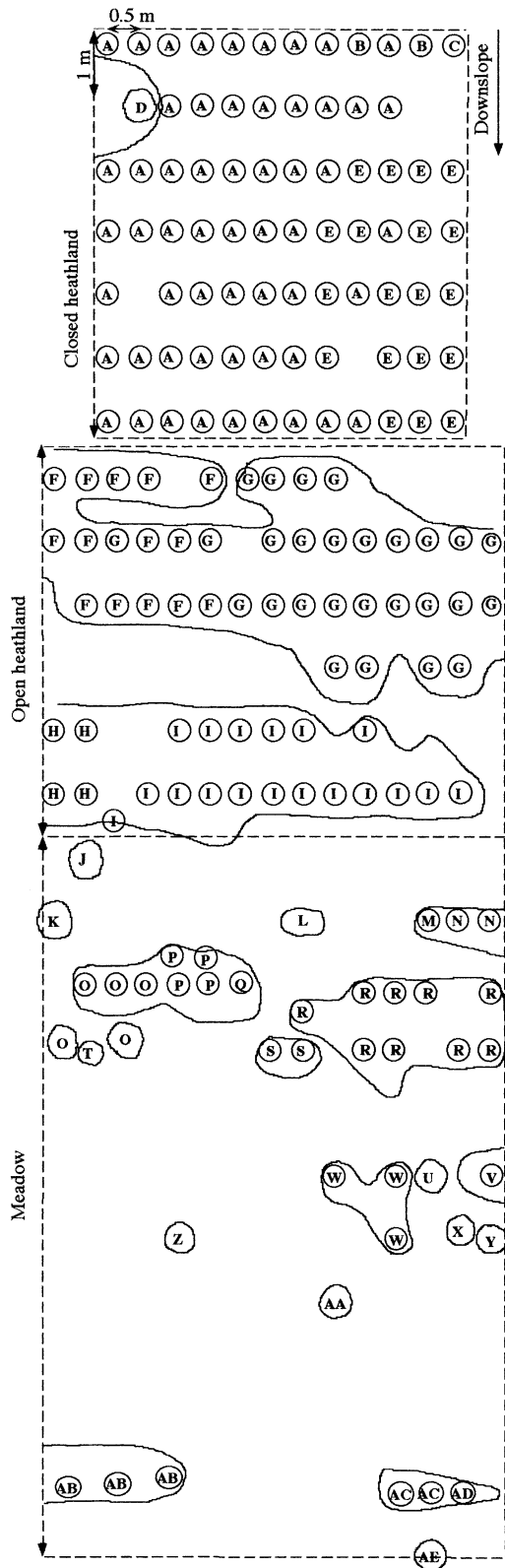


Fig. 1 Spatial distribution of *Rhododendron ferrugineum* genets at the site of Bethmale as revealed by AFLP analysis. Each alphabetic symbol corresponds to a single genet. Continuous lines are limits of heathland patches.

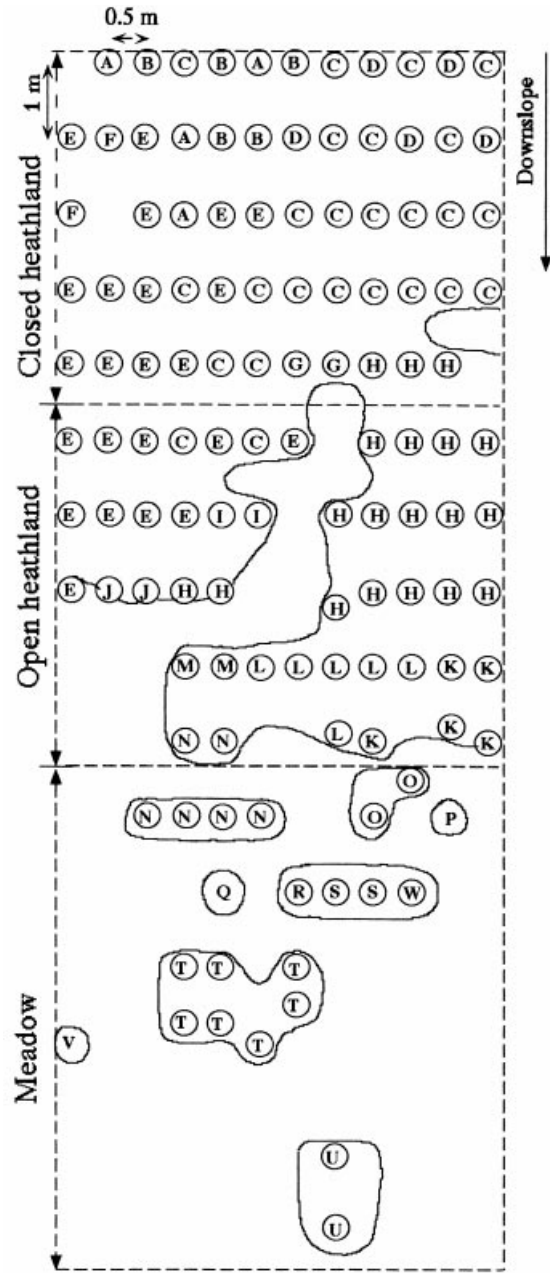


Fig. 2 Spatial distribution of *Rhododendron ferrugineum* genets at the site of Mourtis as revealed by AFLP analysis. Each alphabetic symbol corresponds to a single genet. Continuous lines are limits of heathland patches.

genotype size was nevertheless strikingly smaller at Mourtis than at Bethmale for open and closed heathland. The largest and the smallest genotype sizes are shown in Table 1.

Because most clones in open and closed heathlands were partially outside the sampling plot it was impossible to determine the exact age of most genets in these subplots and

Table 1 Density and main size characteristics of genets in the different subplots at both study sites. Bracketed capital letters correspond to genets mapped in Figs 1 and 2

	Bethmale			Mourtis		
	Meadow	Open heathland	Closed heathland	Meadow	Open heathland	Closed heathland
Genets · m ⁻²	0.27	0.10	0.13	0.26	0.32	0.33
Average genet size (m ² ± SD)	0.52 ± 0.65	6.46 ± 3.46	7.46 ± 10.55	0.63 ± 0.82	2.07 ± 2.81	2.71 ± 3.93
Size of the smallest genets (m ²)	0.02 (Y)	1.96 (H)	0.07 (D)	0.01 (V)	0.84 (I, J, M)	0.84 (F, G)
Size of the largest genets (m ²)*	2.6 (R)	9.73 (G)	25 (A)	2.22 (T)	8 (H)	9.68 (E)

*Values are underestimate due to limited plot size.

Table 2 Age of genotypes in the *Rhododendron ferrugineum* populations estimated from the annual shoot growth rate (ASGR) during the last five years and the spatial extent of clones. > indicates underestimated ages; ASGR are means ± SD. Total length of the genet is the downslope length of aerial part + the average prostrate stem length at the site

Genotypes	Total length (m)	ASGR (cm · year ⁻¹)	Estimated age	Genotypes	Total length (m)	ASGR (cm · year ⁻¹)	Estimated age
BETHMALE							
Closed heathland				O	1.63	1.7 ± 1.08	96
A	> 7.63	2.7 ± 1.29	> 283	P	1.63	2.6 ± 1.36	63
B	> 1.63	2.5 ± 1.26	> 65	Q	1.63	2.0 ± 0.92	81
C	1.13	3.1 ± 1.10	36	R	2.13	3.0 ± 1.88	71
D	> 1.03	1.7 ± 1.11	> 61	S	1.33	3.2 ± 1.19	42
E	> 5.63	3.0 ± 1.17	> 188	T	0.93	1.9 ± 1.02	49
Open heathland				U	0.8	2.9 ± 1.23	28
F	> 2.63	2.8 ± 1.14	> 94	V	> 1.63	2.7 ± 1.57	> 60
G	> 3.63	2.9 ± 1.30	> 125	W	1.63	2.4 ± 1.31	68
H	> 1.63	2.9 ± 1.10	> 56.2	X	1.4	3.0 ± 1.12	47
I	> 2.13	3.8 ± 1.73	> 56	Y	0.93	2.6 ± 1.37	36
Meadow				Z	0.91	2.0 ± 1.12	46
J	1.03	1.5 ± 0.86	69	AA	1.5	1.7 ± 0.82	88
K	0.93	1.6 ± 0.83	59	AB	> 1.45	2.6 ± 1.62	> 56
L	1.03	1.3 ± 0.81	79	AC	1.26	2.3 ± 1.45	55
M	1.13	0.9 ± 0.59	126	AD	1.13	2.4 ± 1.43	47
N	> 1.13	2.1 ± 1.48	> 54	AE	0.85	3.0 ± 1.06	28
MOURTIS							
Closed heathland				L	2.15	2.2 ± 1.22	98
A	> 4.15	3.4 ± 1.46	> 122	M	1.65	2.1 ± 1.05	79
B	> 2.15	3.2 ± 1.40	> 67	N	2.15	3.5 ± 1.01	61
C	> 6.15	4.2 ± 1.79	> 146	Meadow			
D	> 2.15	3.6 ± 1.40	> 60	O	2.1	2.3 ± 1.21	91
E	> 7.15	3.3 ± 1.30	> 217	P	1.6	—	
F	> 2.15	2.1 ± 0.90	> 102	Q	1.55	—	
G	1.65	2.2 ± 1.04	75	R	1.4	1.9 ± 0.56	74
Open heathland				S	2.14	2.5 ± 1.13	86
H	> 4.15	2.7 ± 1.51	> 154	T	2.71	2.4 ± 1.15	113
I	1.65	2.8 ± 0.95	59	U	1.75	1.7 ± 0.87	102
J	1.65	3.2 ± 1.06	52	V	1.55	1.9 ± 0.64	82
K	> 2.15	2.9 ± 1.48	> 74	W	1.65	1.8 ± 0.57	92

the ages shown in Table 2 are underestimated. However, they provide a useful estimation on the time needed for a clone to occupy its current area. The annual shoot growth rates are recorded in Table 2. The average length

of prostrate stem was 0.63 m (±0.31 SD; $n = 26$) and 1.15 m (±0.43; $n = 21$) for Bethmale and Mourtis, respectively. The age of genotype A in Bethmale was estimated to be more than 283 years (Table 2) and the oldest genotype

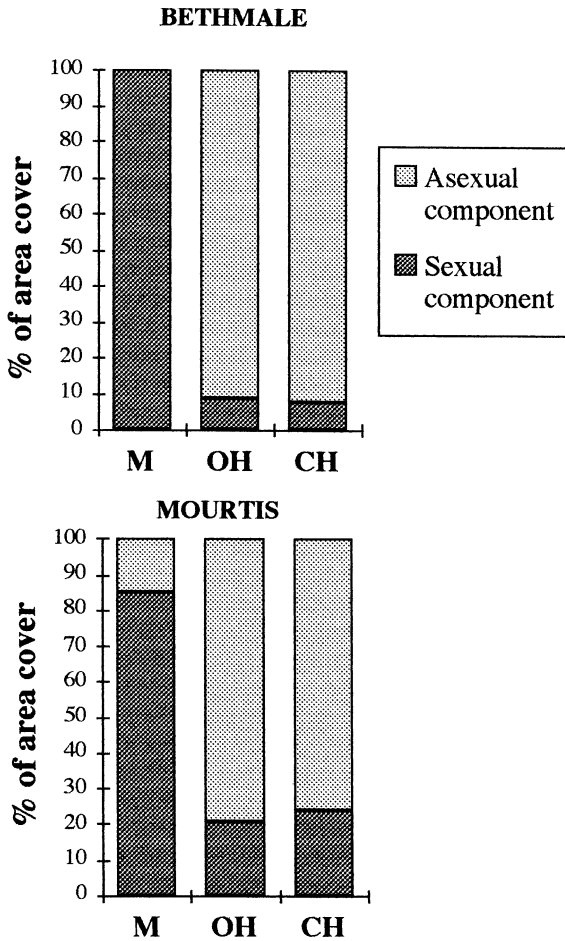


Fig. 3 Sexual and asexual components estimated as a percentage of the total *Rhododendron ferrugineum* population cover in all three successional stages: meadows (M), open heathlands (OH), closed heathlands (CH) of the two sites studied. The sexual component corresponds to the area covered by seed-originated individuals and the asexual component corresponds to the area covered by layering-originated individuals.

was more than 217 years old in Mourtis. The youngest genotype (28 years) was found in Bethmale’s meadow. The youngest genotype of Mourtis was 52 years old and was found in the open heathland.

Taking into account the mean area of nonlayering genets (average size of genets growing in meadow) we deduced the contribution of sexuality and layering to the present population cover in the different subplots of the two sites. Thus, sexual reproduction contributed strongly to *R. ferrugineum* population cover in meadows at the two sites but its contribution was dramatically lower in closed populations particularly at Bethmale (Fig. 3) where the value attained less than 10%.

The mean similarity index between pairs of genets was significantly higher (Mann–Whitney test, $P = 0.0002$) in Bethmale (range from 0.5 to 0.96) than in Mourtis (range from 0.47 to 0.99) (Fig. 4). However, the similarity index changed with population closure (Kruskal–Wallis test, $P = 0.0001$). The Bethmale meadow had a significantly lower mean similarity index (Fig. 5) than open and closed heathland stages, the latter not showing significant differences (Student–Newman–Keuls test, $P < 0.05$). This means that the polymorphism and genotype diversity were lower in both types of heathland than in the meadow. This remained true even when all heathland genotypes from this site were grouped in a single data set and their similarity index compared with that of the meadow (Mann–Whitney test, $P = 0.0001$). At Mourtis, the similarity index also changed with population closure (Kruskal–Wallis test, $P = 0.018$), but the difference was significant only between open heathland and both closed heathland and meadow (Fig. 5).

A common scheme observed at the two sites was that, as population closure increased, the genotypes were much more closely genetically related (Fig. 5). Thus, at Bethmale similarity indexes ranged from 0.64 to 0.96 in

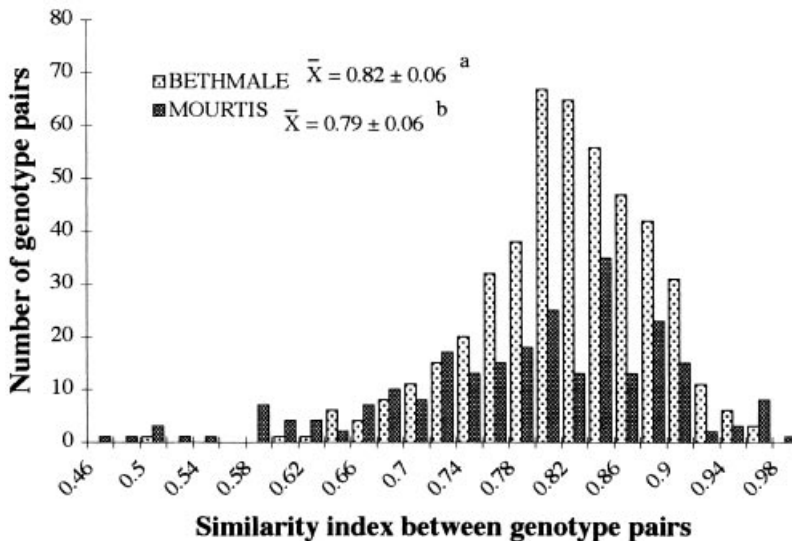


Fig. 4 Frequency distribution of the similarity index calculated for the genet pairs. Different letters indicate that means are significantly different (Mann–Whitney test, $P = 0.0002$).

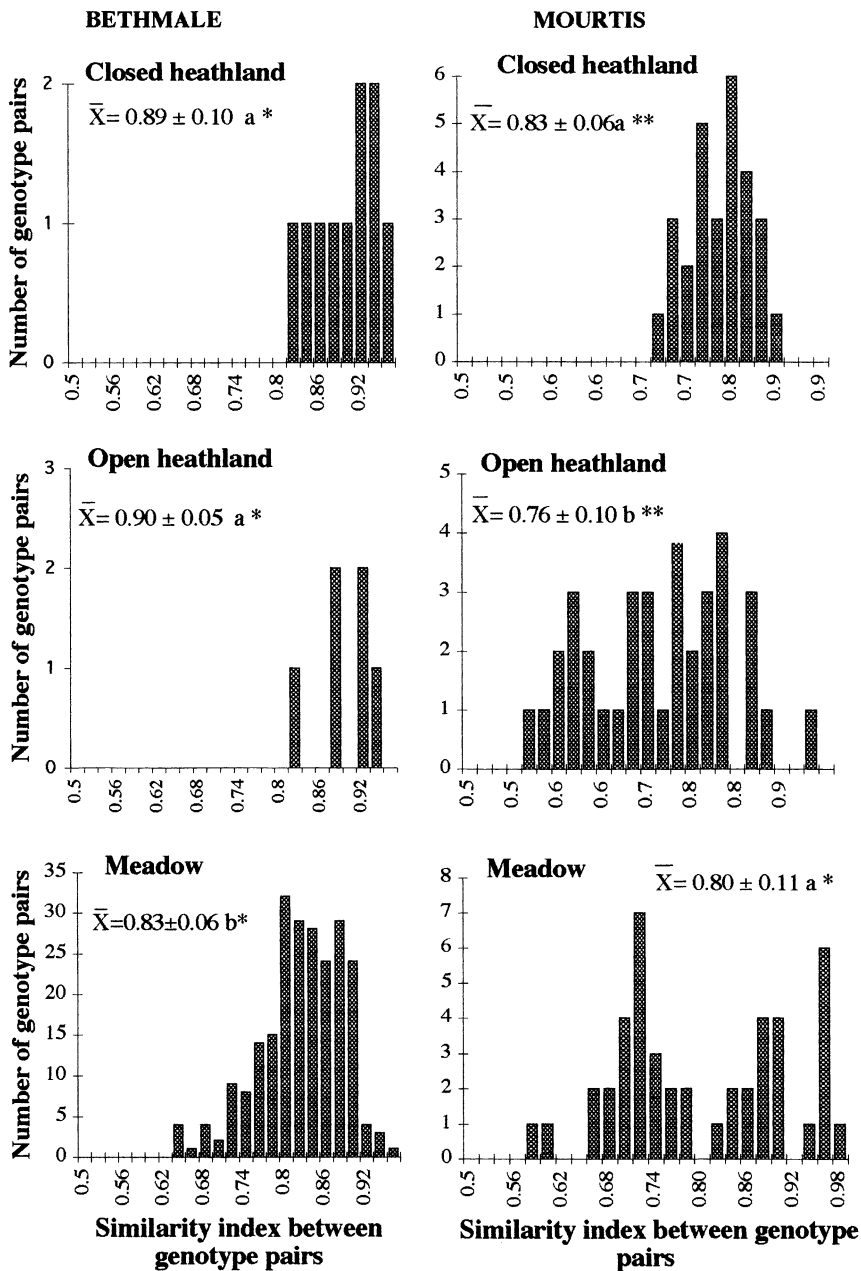


Fig. 5 Frequency distribution of similarity index in each successional stage of the two study sites. Letters indicate a significant difference between means of the subplots within a site (Kruskal–Wallis test followed by a Student–Newman test, $P < 0.05$). Means sharing the same letter are not significantly different. Asterisks indicate a significant difference between means of the subplots of a given successional stage. Means sharing the same number of asterisks are not significantly different. Note that the y-axis scales are not the same.

the meadow and from 0.82 to 0.96 in the closed heathland. The trend was similar at Mourtis where the indexes ranged from 0.58 to 0.99 in the meadow and 0.76–0.90 in the closed heathland. Despite this similar trend, both the open and closed heathlands of Bethmale had a significantly higher mean similarity index than their equivalents in Mourtis (Mann–Whitney test $P = 0.021$ and $P = 0.0006$, respectively). We are not, however, able to detect differences between the meadows at the two sites ($P = 0.140$).

The ‘proportion distinguishable’ genotypes were 0.157 at Bethmale and 0.183 at Mourtis. However, the values

strongly decreased with population closure at both sites (Fig. 6). The decrease was nevertheless more pronounced at Bethmale than at Mourtis.

The estimated genotypic diversity D was 0.85 and 0.93 in Bethmale and Mourtis, respectively. Diversity strongly diminished from meadow to closed heathland in Bethmale, but the decrease was much less pronounced in Mourtis (Fig. 7). Even considering Bethmale’s closed and open heathlands as a single subplot ($D = 0.873$, range from 0.76 to 0.96) the difference with the meadow was still apparent although less pronounced (Mann–Whitney test, $P = 0.0001$).

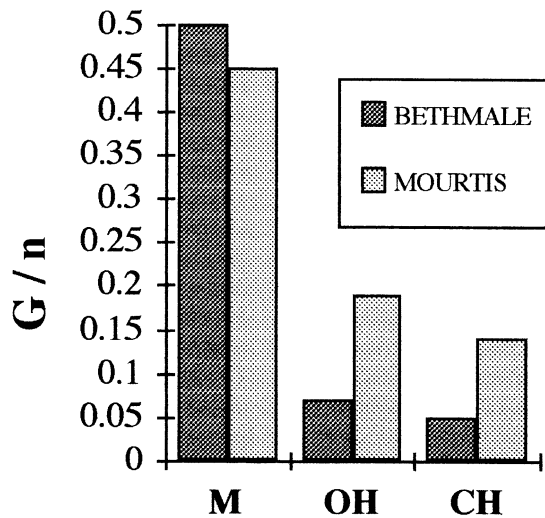


Fig. 6 'Proportion distinguishable' (G/n) genotypes in meadows (M) open heathlands (OH), and closed heathlands (CH) of the two studied sites.

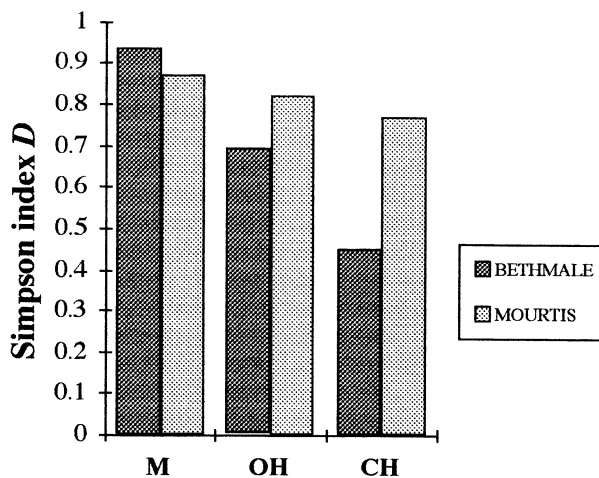


Fig. 7 Genotype diversity assessed by Simpson index D , in meadows (M) open heathlands (OH), and closed heathlands (CH) at the two study sites.

Discussion

AFLP data

AFLP technique has proved to be an efficient tool for identifying genotypes in clonal populations (Escaravage *et al.* 1998) and allowed us to identify 31 (Bethmale) and 23 (Mourtis) multilocus genotypes. This agrees with general findings that clonal populations usually have a high genotypic diversity (Ellstrand & Roose 1987; Hamrick & Godt 1989; Widén *et al.* 1994). Escaravage *et al.* (1998) found 32 genotypes over a 200-m² area in a closed *Rhododendron*

ferrugineum population in the Alps. The diversity reported in the present study (Simpson index: 0.85–0.93) is similar to that given for *R. ferrugineum* in the Alps (0.96, Escaravage *et al.* 1998) and for *Brachypodium pinnatum* (0.83, Schläpfer & Fischer 1998) but higher than for other clonal species (0.62, Ellstrand & Roose 1987; 0.75, Widén *et al.* 1994; 0.61, Godt & Hamrick 1999). The 'proportion distinguishable' genotypes (0.157–0.183) were higher in our populations than those investigated in the Alps (0.08, Escaravage *et al.* 1998) with the same grid. Here, we found high similarity values like those reported in the Alps (0.84, Escaravage *et al.* 1998) which means that the genets were very closely related (Weising *et al.* 1995; Bussemeyer *et al.* 1997).

Despite intermingling of clones in the closed populations, the pattern was not so diffuse that clones could not be delimited. *R. ferrugineum* in mature or young populations is basically a *phalanx* strategist (Pornon *et al.* 1997; Escaravage *et al.* 1998). Despite the fact that the phalanx growth form usually induces a compact clone shape (Jonsson 1995), intermingling in phalangeal genets has already been demonstrated (Schläpfer & Fischer 1998). Owing to the phalangeal architecture of the genet, expanding clones were able to out-compete species growing lower by shading them (Pornon & Doche 1995b).

Origin of genotype structure

In this paper we estimated genet age by average annual length growth of its aerial shoots, and the total length of the genet. The suitability of this method has been demonstrated by the fact that prostrate and aerial branches have similar annual growth rates (Pornon *et al.* 1997). However, because the largest genets were partially outside the sampling area, and because competition between clones may prevent clone extension through various mechanisms (Escaravage 1997), the ages have been underestimated in closed and open heathlands. A more accurate estimate of genet age might show that the closure gradient could account for a temporal colonizing gradient as we first hypothesized. In meadows, one can be more confident with age estimations because genet coalescence has not yet occurred to prevent genet extinction. Therefore, it can be inferred that pioneer genotypes arrived in the Mourtis meadow, 110 years ago mainly during a 40-year period. During this period only nine individuals arrived at the site (a new genet every 4.4 years) and no seedling recruitment occurred during the last 70 years. The dense *Vaccinium myrtillus* population in this subplot competing for resources is possibly responsible for the lack of *Rhododendron* seedling recruitment during the last 70 years, because *Rhododendron* seed germination requires strong light (Bianco & Bulard 1974) and the seeds have low nutrient reserves (Escaravage *et al.* 1997; Pornon *et al.* 1997). For these reasons seedling recruitment in the

Mourtis open and closed heathlands was very unlikely. The 59 and 52-year-old genets (I and J) in open heathland and the smallest one in closed heathland (genotype G) were relics (data not shown) of older genets progressively out-competed by genets E and C extending downslope.

Apparently, the pioneer phase lasted longer in Bethmale. In this site, founders became established about 130 years ago and arrival continued over a 100-year period (one new genet every 4.5 years). We think that older genets growing in meadows were founders because 130 years is less than a single branch can live in the field (165 years, Doche & Pornon 1994) and the shrubs do not appear to have reached their senescence age. Because space free of *Rhododendron* has been occupied by dense *V. myrtillus*-based vegetation, seedling recruitment in open and closed heathlands at Bethmale is unlikely. Genet C, in the closed population was a relic of a larger genet (data not shown). We have no clear idea why seedlings were not recruited during the last 30 years. Summer drought, possibly made worse by global changing climate (Baudière & Gauquelin 1990) could be detrimental to seedling survival.

The results obtained here were consistent with the findings of Pornon & Doche (1995a) showing that, although it produces a considerable number of seeds, meadow colonization by *R. ferrugineum* is a slow process that can take as long as 250 years before total closure is reached. Our results were consistent with the common findings of a low rate of seedling recruitment in clonal populations (Eriksson 1989; Schmid 1990; Steinger *et al.* 1996; Grabherr 1997). Once the pioneer phase was terminated *R. ferrugineum* extended exclusively by layering and the sexual component progressively decreased in the population. As suggested by Pornon & Escaravage (1999) *R. ferrugineum* has neither a strictly ISR nor a strictly RSR behaviour (Eriksson 1989; Jelinski & Cheliak 1992). Seedling recruitment was a repeated process but confined to the early stages of population development. Because seedling recruitment in heathland seemed to be a very rare event, it was highly probable that the current genotypic structure indicates the initial colonization cohort and thus fits the ISR model (Eriksson 1989).

Dynamics of the genotypic structure

The number of genets in a plant population depends on ramet dynamics of existing genets and the recruitment of new genets from seeds (McLellan *et al.* 1997). If seedling recruitment is infrequent in a clonal population and if increasing ramet density leads to the thinning of genets, genotypic diversity in a population is expected to be greatest at the pioneer phase and to progressively diminish with population maturation and closure (Soane & Watkinson 1979; Hartnett & Bazzaz 1985; McLellan *et al.* 1997).

Considering the estimated age in meadows and the underestimation of the oldest clones age from open and closed heathlands, undoubtedly the three stages (meadow, open and closed heathlands), considered in this study, account for three secondary successional stages. Meadow with isolated *R. ferrugineum* represents the pioneer stage of seedling arrival on the site and open heathland, and closed heathland represent increasingly advanced stages in population dynamics.

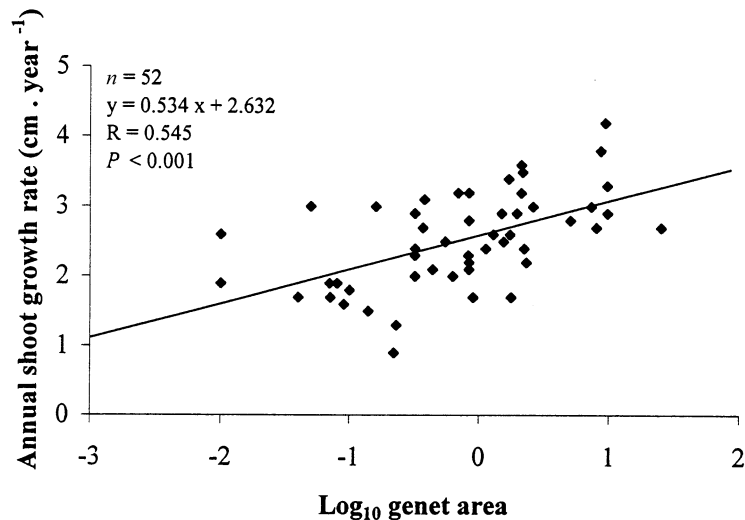
The two sites showed different patterns of genotypic dynamics. At Bethmale, clone area, the density, the 'proportion distinguishable' genotypes, and the genotypic diversity strongly decreased along the closure gradient. The most likely hypothesis is that population closure through ramet production led to a dramatic loss of genets and to a selection of highly genetically related genotypes. Thus, in this site, population closure and maturation could both have reduced genotypic variation (number of genets) and genetic diversity (i.e. allelic; McLellan *et al.* 1997). Clone depletion is very plausible because if we apply the average size of closed heathland genets (7.46 m²) to the meadow subplot (22 genets), only 11 genets would be needed to cover the entire meadow surface (80.5 m²). This means that at least 11 genets would have to be eliminated before a genotypic structure similar to that of the closed heathland is reached.

In contrast at Mourtis, the number of genets and genotypic diversity did not change fundamentally along the closure gradient, even though genets increased in size and 'proportion distinguishable' genotypes decreased. The range of genetic diversity was nevertheless strikingly diminished in the closed population, indicating that thinning could have occurred in this subplot. It is possible that there were more genets in the population at the pioneer phase and that intraspecific competition led to the progressive elimination of less fit genotypes.

One of the most interesting results of this study was that population development seemed to lead to a selection of genetically close genets which has rarely been documented in clonal species. Layering proceeds by rooting of branches growing in litter and progressively covered by soil substrate (Pornon *et al.* 1997). Therefore, it can be hypothesized that if the shoot growth rate is an important trait for successful clonal spread, one would expect that a frequency-dependent selection (Antonovics & Ellstrand 1984; Ellstrand & Roose 1987) would lead to clones with high growth performance becoming steadily predominant in a context of intraspecific competition. In our study, a significant positive correlation between annual shoot growth rate (Fig. 8) and genet area strongly supported the hypothesis that successful clones were favoured during population closure.

There are, however, alternative explanations for genet selection: (i) variable subplot area; Escaravage *et al.* (1998) have shown that the closer the genotypes were located

Fig. 8 Relationships between annual shoot growth and the Log₁₀ area of genets from Bethmale and Mourtis *Rhododendron ferrugineum* populations.



in a population, the closer their genetic relationship. However, subplot areas were not sufficiently different at Mourtis (Open heathland 27.5 m²; closed heathland 24.75 m²) to explain variations of similarity index between the heathland subplots. Even when grouping genets of open and closed heathlands from Bethmale into a single subplot (increase of the sampling area to 77 m² and of the distance between clones) the mean similarity index was always significantly higher in heathland (0.87, range from 0.76 to 0.96) than in meadow (0.83, range from 0.64 to 0.96). (ii) If, in the early stages of closed heathland, pioneers were the founders of a new population and the only source of seeds, then it would not be surprising that their close descendants are genetically related. However, from our knowledge of heathland development, we can assume that the oldest clones from the closed heathlands were only pioneers in a meadow-heathland ecotone (similar to individuals in the current meadow) rather than founders of new populations.

How genotypic structure will change over future time in our closed heathlands is impossible to deduce. Genet intermingling suggests that the genotype pattern is not definitely fixed and it is possible that the smallest clones will disappear in the near future. However, results of Escaravage *et al.* (1998) suggest a long coexistence of clones in an old *R. ferrugineum* population. Although ranging over a much longer period, genet dynamics in *Rhododendron* could be fairly similar to that in *Solidago canadensis* studied by Hartnett & Bazzaz (1985). In this species seedling recruitment was limited to a pioneer stage (3 years) followed by a stage of loss of the youngest and smallest genets. In a 15-year-old field genet coexistence seemed ensured for at least a 6-year period.

Several hypotheses have been formulated to account for the mechanisms underlying the maintenance of a high level of genotypic diversity in clonal species: (i) icrosite

heterogeneity which promotes the coexistence of clones through diversifying selection (Antonovics & Ellstrand 1984; Ellstrand & Roose 1987); (ii) occasional seedling recruitment (Soane & Watkinson 1979); and (iii) symmetric competition among clones (Hartnett & Bazzaz 1985). Because we carefully assessed habitat homogeneity in our study, and because the shrub is able to overcome habitat heterogeneity, the first hypothesis alone is insufficient to explain genotypic arrangement. In the last section we concluded that seedling recruitment in a closed population can be excluded. The third hypothesis could be more promising and deserves to be investigated. When clone coalescence occurred through ramet production, frequency-dependent selection favoured the fittest genets, i.e. those having high growth performance. As a consequence, symmetric competition (de Kroon *et al.* 1992) between clones might occur progressively which could have allowed the clonal structure to be conserved until now. Obviously this scenario would need more investigation to be proved.

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