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Establishment and genetic structure of *Empetrum hermaphroditum* populations in northern Sweden

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Abstract. In late successions of the boreal forest of northern Sweden the evergreen dwarf shrub *Empetrum hermaphroditum* forms an extensive cover and is believed to spread mainly vegetatively through layering. To analyse the process of population establishment and the relative importance of sexual vs asexual reproduction and the spatial clonal distribution of this species we selected one mainland and two island sites of different post-fire successional ages (145, 375 and 1720 yr since last fire, respectively). Using 61 polymorphic RAPD markers, we found 96 genotypes in a total of 133 samples. All three populations showed high levels of genetic variation. AMOVA analysis revealed that 33% of the total variation resided among sites, 26% resided among plots within sites and 41% was due to variation within plots. The youngest population had only 14% clonal fraction. In contrast, the oldest population had > 30% clonal fraction and many genets had dimensions of 10-40 m and were intermingled. It appears that *E. hermaphroditum* establishes by seeds to a larger extent than previously thought and that the clonal spread by layering is rather slow.

Keywords: Clonal structure; Crowberry; Fire history; Genetic variation; Layering; RAPD.

Nomenclature: Haapasaari (1988).

Abbreviation: PCR = Polymerase Chain Reaction; RAPD = Random Amplified Polymorphic DNA.

Introduction

In environments with strong abiotic influence such as alpine, arctic and boreal areas, vegetative propagation plays an important role in terrestrial plant reproduction as more than 90% of the plant species are clonal and spread vegetatively (Bliss 1971). The extent of clonality affects the mating system, population structure and fruit set (Hermanutz et al. 1989). *Empetrum hermaphroditum* (crowberry, *Empetraceae*), is a long-lived, monoecious evergreen dwarf-shrub, growing in temperate and polar biomes in northern Europe, Asia and North America. The species has a circumpolar distribution, and is found extensively across the boreal zone of Fennoscandia (Callaghan & Emanuelsson 1985; Hultén & Fries 1986; Haapasaari 1988). With prolonged absence of wildfire on mesic and nutrient poor forest sites in northern Sweden, *E. hermaphroditum* often becomes dominant in the ground layer vegetation where it forms extensive and dense mats (Nilsson 1992; Wardle et al. 1997). The mode of reproduction and the establishment pattern of *E. hermaphroditum* are not well understood. Despite abundant seed production, field observations of seedlings are rare at undisturbed sites in late successions (Bell & Tallis 1973; Eriksson 1989) and the species is thought to mainly spread vegetatively through layering (Eriksson 1989). Plant establishment by seeds is more commonly observed on forest sites where the mineral soil has been exposed or on decomposing logs or stumps (Marie-Charlotte Nilsson pers. obs.). *Empetrum hermaphroditum* is highly susceptible to wildfire due to its superficial root system and prostrate growth habit, and often completely disappears after a fire (Uggla 1958; Vakurov 1975). The species seldom produces a thick stem or easily observable 'centre' and it does not form rhizomes. This growth habit makes it difficult to delimit

individual plants and to differentiate ramets from genets, thus precluding the study of genotypic structure and population dynamics.

Molecular markers have been widely used to characterize clones and the mode of reproduction in plants and represent the most suitable ways of identifying genotypes and ramets that undergo both sexual and vegetative reproduction (Parker & Hamrick 1992; Parks & Werth 1993; Ayres & Ryan 1997; Esselman et al. 1999; Wang & Szmidt 2001). The advantage of random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) is the potential to offer a high number of polymorphic loci that can be used to tag genotypes. The technique is simple, fast and economic. The high degree of polymorphism of the RAPD markers makes it possible to identify asexually produced clones without ambiguity (Stiller & Denton 1995; Wang 1997; Arens et al. 1998; Gabrielsen & Brochmann 1998; Bushakra et al. 1999). The aims of this study were to analyse the population genetic variability, to discriminate and delineate clones and thereby determine the relative importance of sexual and vegetative reproduction and patterns of clonal spread of *E. hermaphroditum* on sites of different successional stages. We hypothesize that abundant seed production indicates that the sexual reproduction is more important for the species' spread and reproduction than clonal propagation through layering.

Material and Methods

Study sites and sampling procedure

Three locations in northern Sweden, one mainland site (Rackträsk) and two islands (Åskön, Pollenön), of different post-fire successional age, were selected for the present study (Table 1). The two islands are located on Lake Hornavan and are separated by 3.5 km. The islands were formed during the early Holocene from glaci-fluvial material and are 20 km from the mainland site. Previous disturbance by fire was determined by dating of fire scars (Zackrisson 1977) and by AMS ^{14}C dating (at the Tandem Laboratory, Uppsala, Sweden) of charcoal particles sampled from the uppermost burned (charcoal) layer in organic soils at each of the investigated site (Zackrisson et al. 1996). Investigation of vegetation composition was conducted for all the sites and the ecological characteristics of the three sites are presented in Table 1. We should point out that our sampling of sites is limited and the lack of replications for each age class would not permit a proper synthesis on post-fire population development.

In the absence of prior knowledge of the extent of *E. hermaphroditum* layering on different forest sites, we set up five plots on each site and within each plot samples were collected systematically by grid, to ensure both closely and distantly located samples from each site. Five 2 m × 2 m square plots were selected at 20-m

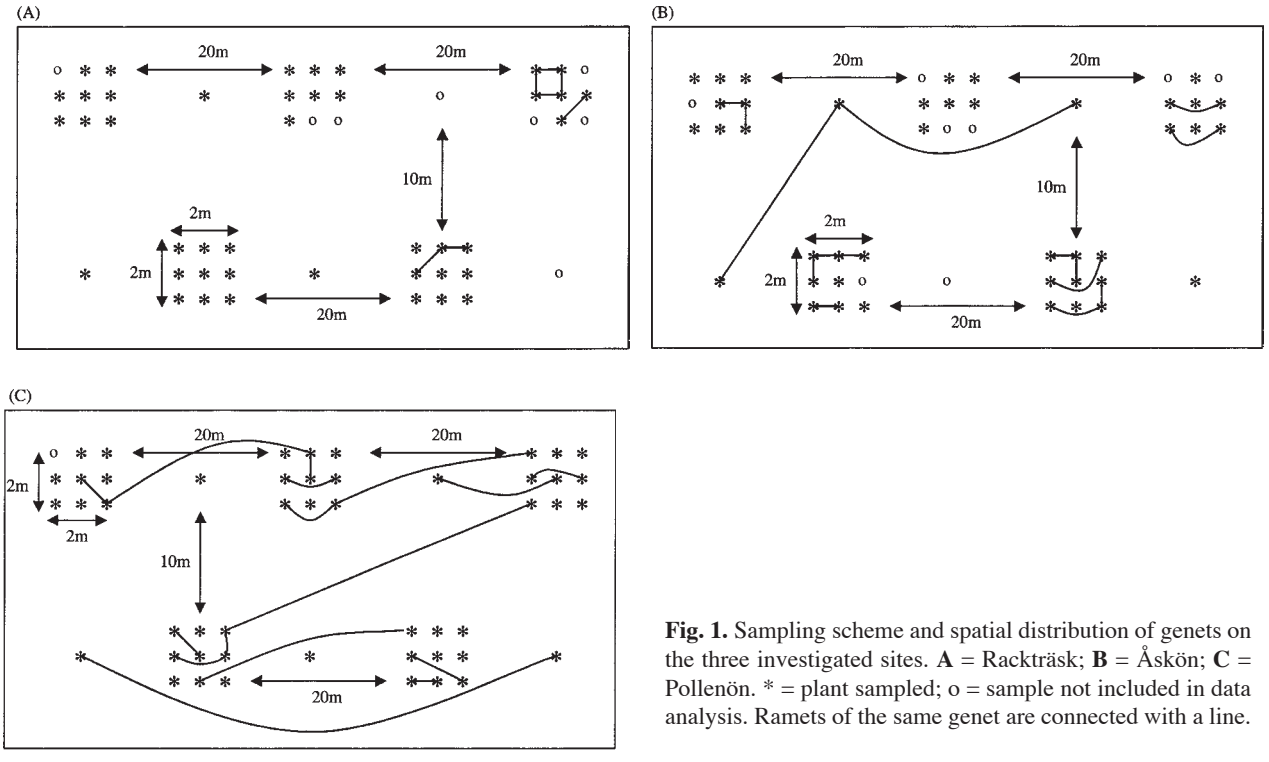

Fig. 1. Sampling scheme and spatial distribution of genets on the three investigated sites. **A** = Rackträsk; **B** = Åskön; **C** = Pollenön. * = plant sampled; o = sample not included in data analysis. Ramets of the same genet are connected with a line.

Table 1. Ecological characteristics of the investigated sites.

Site		Rackträsk	Åskön	Pollenön
Years since last fire		145	375 ± 55	1720 ± 60
Elevation (m a.s.l.)		434	427	427
Coordinates		66°01'73 N	66°08'01 N	66°09'67 N
		18°03'61 E	17°48'71 E	17°47'08 E
Humus thickness (cm)		3.0 ± 0.6	8.0 ± 1.2	40.3 ± 4.7
Tree basal area (m^2ha^{-1})	*Pinus sylvestris*	19.3 ± 0.8	16.3 ± 0.3	0
	Picea abies	0	0.7 ± 0.3	0
	Betula spp.	0	0	2.3 ± 0.9
Cover (%) of dominant field layer species	*Empetrum hermaphroditum*	20.0 ± 0.8	48.3 ± 4.3	70.0 ± 2.9
	Vaccinium vitis-idaea	16.7 ± 1.7	33.3 ± 1.7	5.0 ± 0.0
	Vaccinium myrtilllus	1.3 ± 0.3	2.7 ± 1.7	4.0 ± 0.0
	Vaccinium uliginosum	0	0	13.3 ± 1.2
Cover (%) of dominant bottom layer species	*Cladina* spp.	51.7 ± 4.4	1.7 ± 0.3	0
	Pleurozium schreberi	5.7 ± 0.7	61.7 ± 6.0	71.7 ± 6.0
	Dicranum spp.	16.7 ± 1.7	5.7 ± 2.3	6.7 ± 1.7
	Hylocomium splendens	0.3 ± 0.3	18.3 ± 0.6	1.0 ± 0.6

intervals along two parallel transects (separated by 10 m) with random starting point on each site. Nine evenly distributed samples were collected at 1 m intervals in each plot. In addition, one sample was taken at each transect midpoint separating the plots. A short branch with the current year foliage was taken at each sampling point. A total of 50 samples were collected at each site; a total of 150 samples from the three locations (Fig. 1).

DNA extraction and RAPD analysis

Genomic DNA was extracted from leaves following the method of Doyle & Doyle (1990). PCR amplifications were carried out in a thermal cycler PTC-100 (MJ Research Inc., Waltham, MA, USA) programmed for an initial denaturation of 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. The primer extension step at 72 °C of the final cycle was extended to 5 min. The RAPD reaction mix consisted of 10 ng of DNA, 150 µM of each dNTP (Pharmacia, Uppsala, SE), 0.2 µM of primer and 0.5U of Taq DNA polymerase (Pharmacia), 1.5 mM $MgCl_2$, in a total volume of 25 µl. PCR products were resolved by electrophoresis on 1.4% agarose gels in 0.5 × TBE buffer. The gels were stained with ethidium bromide and RAPD profiles were visualized under UV light. One kilobase ladder (GIBCOBRL, Life Technologies, Calsbad, CA, USA) was used as DNA size standard.

Primers used in this experiment were 10-base random primers from Operon Technologies (Alameda, CA, USA). In the first step of the analysis, 40 primers (OPA01-20 and OPX01-20) were screened on 20 samples, to select for primers that yield strong and polymorphic RAPD profiles. Of the 40 primers tested, seven (OPA-18, OPX-4, OPX-13, OPX-20, OPZ-3, OPZ-6 and OPZ-7) gave good amplification profiles. To verify the reproducibility of the RAPD profiles generated by these seven primers, amplifications were carried out twice for a group of 20 samples. Once satisfied, the seven primers were used in the analysis of RAPD variation in the 150 samples collected from the three sites.

Data scoring and statistical analysis

Amplified fragments, named by the primer used and the molecular weight in base pairs (bp), were scored as presence (1) or absence (0) of a fragment. Fragments were scored by two persons independently and then compared. Uncertain bands were discarded. Each distinct and reproducible RAPD fragment was regarded as a putative locus. In the data analysis, only the polymorphic loci were used. Allele frequencies were computed using the Lynch & Milligan's (1994) Taylor expansion method, as implemented in the TFPGA program (Miller 1997). By using allele frequencies, the probability that each multilocus genotype could be produced in each population and their second encounter were calculated according to Parks & Werth (1993), assuming selfing.

Genotypic diversity was quantified by a normalized Shannon's diversity index (H_s) as described in Goodwin et al. (1992):

$$H_s = -\Sigma P_i * \ln P_i / \ln N, \quad (1)$$

where P_i is the frequency of the i-th multilocus genotype and N is the sample size. The values for H_s range from 0 to 1, and the maximum possible value occurs when each individual in the sample has a different genotype. We also calculated Simpson's diversity index modified for finite population size (Pielou 1969):

Table 2. Diversity measurements for the investigated populations of *E. hermaphroditum*. H_s = genotypic diversity; d = Simpson's index of diversity.

Population	Sample size	Polymorphic loci	Genets sampled 1 ×	Genets sampled > 1 ×	Total	Clonal fraction	H_s	d	Proportion distinguishable
Rackträsk	42	52 (85%)	33	3	36	14.3%	0.935	0.988	0.857
Åskön	42	47 (77%)	18	9	27	35.7%	0.845	0.974	0.643
Pollenön	49	47 (77%)	22	11	33	32.7%	0.866	0.980	0.673
Total	133	61 (100%)	73	23	96	27.8%	0.908	0.994	0.722

$$d = 1 - \Sigma\{(n_i(n_i - 1))/(N(N - 1))\}, \quad (2)$$

where n_i = number of samples with genotype i and N is the total number of samples; and an additional measure of clonal diversity, by Ellstrand & Roose (1987) termed 'proportion distinguishable', which is the number of genets (G) relative to the number of samples (N). The clonal fraction, i.e. the proportion of samples derived from clonal propagation, was calculated for each population as $(N - G)/N$.

The differences among multilocus genotypes were measured by genetic distance calculated as: $D = n - n_{xy}$, where n is the total number of polymorphic fragments and n_{xy} is the number of fragments shared by two genotypes. A distance matrix was generated for all possible pairwise comparisons among different genotypes in each population. Apportionment of genetic variation at three hierarchy levels, among sites, among plots within sites and within plots, was made using the analysis of molecular variance (AMOVA, Excoffier et al. 1992). In this analysis, the between-plots additional samples were removed. Levels of significance for variance components were calculated by a Monte-Carlo procedure using the WINAMOVA program (Excoffier et al. 1992), with 1000 permutations. The spatial metric distance matrix between every two samples within each population was compared with the genetic distance matrix. The correlation between the two matrices was analysed using a Mantel matrix-correspondence test (Sokal & Rohlf 1995). A significance test for the correlation coefficient (r) was obtained using the standard normal variate (g). The Mantel test was performed using the program MANTEL v2.0 (Liedloff 1999).

Results

Due to poor quality of some of the PCR products and/or DNA samples, 17 samples were excluded from the data analysis. The final data set included 133 samples and 61 polymorphic loci for the seven primers used. The number of loci detected by individual primers varied between five for the OPZ-6 primer and 11 for the OPX-20 primer. The size of RAPD bands scored by individual primers ranged between 2500 and 300 bp. Of the 61 loci examined, 52 (85%) loci were polymorphic in the mainland population Rackträsk, while 47 (77%) loci were polymorphic in the island populations Åskön and Pollenön (Table 2).

When genotypes at each of the 61 loci were combined, a total of 96 different multilocus genotypes (genets) were identified among the 133 samples. Among these 96 genotypes, 73 (76%) genotypes were detected only once, and the remaining 23 (24%) genotypes were detected more than once (Table 2). The majority of the genotypes were found to be very different from each other; 45-52% of the genotypes differed by 21-30 fragments, less than 12% pairs of genotypes differed by 1-10 fragment(s). The maximum observed difference between two 61-locus genotypes was 44 fragments. For the 23 shared genotypes we calculated the genotype probability in the population they occurred. Since little is known about the mating system of the species, we assumed selfing in this estimate. This would give a minimum estimate. The genotype probability for each of the 23 multilocus genotypes is less than 10^{-15} and the

Table 3. Results of analysis of molecular variance (AMOVA). Between plots additional samples at each site were not included in this hierarchical partition, which leaves a total sample size of 121.

Source of variation	*df*	Sum of squares deviation	Mean squares deviation	Variance components	*P*-value*
Among sites	2	406.475	203.237	4.235 (32.7%)	< 0.001
Among plots within sites	12	388.383	32.365	3.357 (25.9%)	< 0.001
Within plots	106	570.300	5.380	5.380 (41.5%)	< 0.001

*Probability of having a more extreme variance component than the observed value by chance, computed by Monte-Carlo procedure from 1000 random permutations.

probability of their second encounter in a population is less than 10^{-13} (data not shown). Thus, samples sharing identical multilocus genotypes were regarded as ramets of the same genet.

Values for the genotypic diversity index H_s and Simpson's diversity index d for the investigated populations were high and ranged between 0.85 and 0.94, and 0.97 and 0.99, respectively (Table 2). The 'proportion of distinguishable' ranged from 0.64 to 0.86, with the highest value in the mainland site Rackträsk. The clonal fraction in the whole sample set (three populations) was 28%. The lowest value (14%) was found for the mainland population Rackträsk. In contrast, both island populations Åskön and Pollenön contained much larger clonal fraction of 36% and 33%, respectively (Table 2). Partition of the total genetic variation by AMOVA revealed that 33% of the total variation were attributable to differences among sites, 26% to differences among plots within sites and 41% to differences among samples within plots (Table 3).

The spatial distribution of the clonal individuals identified differed among the three populations studied. In the youngest population Rackträsk only three of the 36 genets were found to have more than one ramet sampled (two, three and four ramets, respectively for the three genets) and all the ramets of the same genet were located within 2 m of distance from each other (Fig. 1a). No genets were shared among plots on this site. The middle-aged population Åskön had nine genets with more than one ramet sampled, of which four had two ramets, another four had three ramets and another one had four ramets. Eight of these genets had their ramets within the same plots. Only one genet had its three ramets spread over a distance of 30 m (Fig. 1b). On the other hand, in the late successional population Pollenön more large size genets were detected. Of the 11 genets with more than one ramet detected on island Pollenön six were spread over large distances ranging from 10 to 40 m. Furthermore, ramets belonging to different genets intermingled considerably and several genets were shared among different plots (Fig. 1c). There was no genet shared among the three populations.

The Mantel test of the correlation between spatial distances and genetic distances revealed a higher correlation ($r = 0.337$, Table 4) in population Åskön. Correlation in populations Pollenön and Rackträsk was lower with $r = 0.187$ and 0.211, respectively. Significance test using the standard normal variate indicated that in all three populations the null hypothesis of no association between spatial distances and genetic distances should be rejected (Table 4).

Discussion

Empetrum hermaphroditum is a slow-growing, late-successional species, for which both sexual and asexual propagation allows colonization of vacant sites. It can take 100 yr or more before it dominates the site (Nilsson 1992). The present study revealed significant diversity among samples from all three populations. As many as 96 different multilocus genotypes were identified, among which 76% were detected once and only 24% were detected more than once in the 133 samples analysed. Genotypic diversity ($H_s = 0.85$-0.94) and Simpson's diversity index ($d = 0.97$-0.99) were high in all populations. This observed level of diversity in *E. hermaphroditum* populations was above the mean values of $d = 0.62$ for other clonal species reviewed by Ellstrand & Roose (1987). This indicates that clonal propagation was not the main factor contributing to the population composition, especially not in the young populations where clonal fraction was low. The clonal fraction in all the three sampled populations was only 28%. The genetic distances among individual genets were generally large indicating that most of the genotypes detected in the investigated populations were not closely related. The observed large number of unique genotypes and the fact that most of the clones had very few ramets suggest that the majority of the samples from these three locations are results of sexual reproduction and that the initial colonization and establishment of *E. hermaphroditum* populations are predominantly facilitated through seeds. Seedling recruitment during the succession will tend to increase and maintain population genetic diversity.

In this respect *E. hermaphroditum* resembles other species for which vegetative propagation does not substitute for sexual reproduction, but rather serves as a

Table 4. Results from the Mantel test of the comparison of genetic and spatial metric distance matrices.

Site	Correlation coefficient r	Standard normal variate g	Critical value for g	Conclusion
Rackträsk	0.211	5.672	$g_{0.05} = 1.645$	$g >$ critical value,
Åskön	0.377	8.131	$g_{0.01} = 2.326$	reject Ho*
Pollenön	0.187	4.595		

*Null hypothesis: no association between spatial distance and genetic distance matrices.

means for horizontal spread of sexually reproduced individuals. Similar to the results obtained in this study populations of other clonal species have been found to maintain significant genetic variation despite possibly reduced effective population sizes (Gabrielsen & Brochmann 1998; Mayes et al. 1998). It appears that clonal reproduction does not constrain the maintenance of genetic diversity in *E. hermaphroditum* at the population level. Even small amounts of sexual recruitment can maintain genetic diversity. Clonal growth may also contribute to the maintenance of genetic variation by increasing the life span of rare genotypes and by preserving heterozygous genotypes. These traits may have been of importance for *E. hermaphroditum* to re-occupy the large variety of boreal and arctic sites produced by repeated Pleistocene ice age disturbances in the past (Huntley & Webb 1988).

Little is known about the mating system of *E. hermaphroditum*. The species is believed to be mainly wind-pollinated (Good 1927) which is likely to facilitate outcrossing. Abundant flowering and seed production are common in the region of our sampling (Grime et al. 1988). Seeds are dispersed by birds (Grime et al. 1988) and possibly travel long distances. This breeding system will tend to maintain genetic diversity of individuals and populations. Seed dispersal by birds can cause patchy distribution of genotypes and higher differentiation among sites. In addition, family structure and clonal propagation will also increase the differentiation level among sites. AMOVA analysis revealed high variance at all three spatial scales suggesting that different genotypic compositions of the founder populations, fine scale genetic structure and clonal reproduction all played an important role in forming the present genetic structure of the three populations investigated. The observed value of population differentiation (32%) in *E. hermaphroditum* is much higher compared to the outcrossing and wind pollinated main forest conifer tree species *Picea abies* and *Pinus sylvestris* in northern Sweden, which usually have a population differentiation level of 5% (Wang & Szmidt 2001).

As demonstrated in the present study, the relative importance of sexual and asexual reproduction varied among sites. The mainland population, which experienced fire disturbance only 145 yr ago, harboured the largest number of distinct genets and a small clonal fraction. In contrast, the two late successional island populations, which had not experienced fire disturbance over the last 375 and 1720 yr had much larger (> 30%) clonal fractions. The clones in the oldest population (Pollenön) were much larger than the ones in the middle-aged population at Åskön. These results may suggest that in the first 100-300 yr after fire the development of *E. hermaphroditum* populations on the burned sites was mainly facilitated through seeds and clonal development was limited. During this time, fine scale family structure may form through seeds produced by the existing individuals on sites. The Mantel test indicated that there was a positive correlation between genetic and spatial distances among samples in each of the three populations suggesting that individuals growing in a close proximity tend to be more related genetically. The highest correlation (r = 0.38) was found on the middle-aged population at Åskön. The two other populations had lower r-values of 0.21 and 0.19, respectively. This trend reflects the pattern of *E. hermaphroditum* population development. When a population was first established by random genotypes through seed migration there should be little correlation between genetic distances and spatial distances between individuals. As the population develops, seedling recruitment over a short distance from the mother plants may create fine-scale family structure within a few m, which would result in a higher r-value. In addition, small clustered genets developed in the middle-aged populations would also increase the r-value. As asexual reproduction becomes more important at late successional stage and genets become much spread and intermingled, the spatial family structure would be disrupted, thus, in turn decreasing the r-value. However, our present study was designed to provide information about the relative importance of sexual and asexual reproduction of the species rather than on the precise follow-up of the post-fire population development. Our sampling of sites is limited to derive a full understanding of the latter process. The proposed relationships should be tested in a study with replicated sites in sufficient numbers. The youngest population in this study was 145 yr old and the age intervals to the other two populations (375 and 1720 yr old) are large. Additional younger (less than 100 yr) populations and more intermediate-aged populations between our sampled three populations would be desirable to better monitor the population development in time and the effect of fire.

Once established, *E. hermaproditum* produces foliage and litter of poor quality with high levels of secondary metabolites (Nilsson et al. 1998; Wardle et al. 1998), which are known to negatively influence seed establishment of other species (Zackrisson & Nilsson 1992; Zackrisson et al. 1995). It is likely that an increased vegetative reproduction of *E. hermaphroditum* on late-successional sites may compensate for possible autotoxicity of germinating seed on sites dominated by the species. The root formation on layering branches and the adaptability of symbiotic ericoid myccorhizae to tolerate toxic compounds such as phenolics in the soil (Smith & Read 1997) may further explain why vegetative spread becomes more dominant at later successional stages. *E.*

hermaphroditum produces a relatively large seed bank (Komulainen et al. 1994). However, new seedlings are seldom observed in thick carpets of *E. hermaphroditum*, and seeds are difficult to germinate under laboratory conditions (Bell & Tallis 1973). The seed germination mechanisms of *E. hermaphroditum* are poorly understood but the predominant sexual reproduction on early successional sites could simply be explained by favourable microsite conditions with a higher pH, reduced autotoxicity of phenols by the presence of charcoal (Zackrisson et al. 1996) and/or reduced competition from surrounding vegetation.

According to the spatial distribution of *E. hermaphroditum* genets in the early successional mainland population, the clones appear to be restricted in extent and contain only clustered ramets with no inter-growth. On the other hand, the two older populations exhibit different clonal architecture. Firstly, some genets have achieved considerable dimensions of up to 40 m. Secondly, ramets from different genets were frequently intermingled. Prior to this study, the size range of *E. hermaphroditum* clones was unknown, although estimates of up to 2 m had been made based on the sizes of discrete patches that occur at the northern limit of species distribution (Elvebakk & Spjelkavik 1995). Our present results suggest that, especially at sites with long fire return intervals, these previous estimates were conservative. The small mean number of ramets per genet suggests that the development of larger (> 10 m) genets takes hundreds of years. The age of the genets on the investigated sites is not known. However, *E. hermaphroditum* individuals as old as 80 yr have been reported to occur (Elvebakk & Spjelkavik 1995), but the maximum age is probably much higher. Furthermore, the fact that the widespread genets detected in this study contained small numbers of ramets indicates that expanding genets often become fragmented as a result of competition with other genets and repeated establishment of new genets by seed. With the sampling scheme employed in this study, genets separated by less than 1 m would go undetected. It is, therefore, possible that our results underestimate the actual clonal fraction. Nevertheless, while additional ramets may be present in the investigated populations the observed high genotypic diversity indicates that sexual reproduction was the main component in setting up the populations. It appears that *E. hermaphroditum* establishes by seeds to a larger extent than was previously thought and that clonal spread is relatively slow. More studies are required to determine whether predominant sexual reproduction revealed in the present study is a general feature of the species within its main distribution.

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