
Implications of Clonal Structure for Effective Population Size and Genetic Drift in a Rare Terrestrial Orchid, *Cremastra appendiculata*

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Abstract: *Effective population size (N_e) influences the degree to which random genetic drift changes allele frequencies, increases inbreeding, and decreases genetic diversity, and thus is a parameter of direct relevance to the conservation of rare species. Few empirical data are available, however, concerning the effects of clonal structure on N_e in plant species reproducing both sexually and asexually. Using genetic markers and spatial autocorrelation analysis, we quantified the statistical significance and spatial scale of clonal spread of six populations of the rare terrestrial orchid *Cremastra appendiculata* (D. Don) Makino in a large (180 ha), undisturbed landscape on Oenaro Island, located off of the southeastern coast of South Korea. We used this information to calculate three demographic estimators of N_e : the number of ramets within a population, the number of genets, and an estimator that incorporates information on both the number of genets and variation in the number of ramets per genet. Taking clonal structure into account results in up to a fivefold decrease in estimates of N_e relative to the ecologically apparent number of individuals within populations. Levels of standing genetic variation are in fact greater than expected given our estimates of N_e , leading us to consider historical factors resulting in N_e being greater in the past than in present-day populations. Like *C. appendiculata*, many terrestrial orchids occur in relatively small, spatially isolated populations and are of special concern for conservation. Our results indicate that efforts aimed at the long-term preservation of these species should be based on a sound understanding of the potential for clonal structure and its implications for the sensitivity of populations to losses of genetic diversity and fitness via random genetic drift.*

Key Words: allozymes, clones, *Cremastra appendiculata*, effective population size, fitness, Orchidaceae, spatial genetic structure

Implicaciones de la Estructura Clonal para el Tamaño Poblacional Efectivo y la Deriva Génica en una Orquídea Terrestre Rara, *Cremastra appendiculata*

Resumen: *El tamaño poblacional efectivo (N_e) influye en la proporción en la que las frecuencias alélicas son cambiadas por deriva génica aleatoria y disminuye la diversidad genética, y por lo tanto es un parámetro de relevancia directa para la conservación de especies raras. Sin embargo, existen pocos datos empíricos sobre los efectos de la estructura clonal sobre N_e en especies de plantas de reproducción sexual como asexual. Con el uso de marcadores genéticos y análisis de autocorrelación espacial cuantificamos la significación estadística de la expansión clonal de seis poblaciones de la orquídea terrestre rara *Cremastra appendiculata* (D. Don) Makino en un paisaje extenso (180 ha) no perturbado en la Isla Oenaro, localizada cerca de la costa sur de Corea del Sur. Utilizamos esta información para calcular tres estimadores demográficos de N_e : el número de ramiúlas en una población, el número de "genets" y un estimador que incorpora información tanto del número de "genets" y la variación en el número de ramiúlas por "genet." Tomando a la estructura clonal en consideración resulta en un decremento de hasta cinco veces en las estimaciones de N_e en relación con el*

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número ecológicamente aparente de individuos en las poblaciones. De hecho, los niveles de variación genética son mayores al esperado dadas nuestras estimaciones de N_e , llevándonos a considerar factores históricos que resultan en N_e sea mayor en poblaciones del pasado que en las actuales. Como *C. appendiculata*, muchas orquídeas terrestres ocurren en poblaciones relativamente pequeñas, aisladas espacialmente y son de especial preocupación para la conservación. Nuestros resultados indican que los esfuerzos encaminados a la preservación de estas especies a largo plazo deben fundamentarse en un profundo entendimiento del potencial de la estructura clonal y sus implicaciones para la sensibilidad de poblaciones la pérdida de diversidad genética y adaptabilidad por deriva genética aleatoria.

Palabras Clave: adaptabilidad, alozimas, clones, *Cremastra appendiculata*, estructura genética espacial, Orchidaceae, tamaño poblacional efectivo

Introduction

The degree to which random genetic drift changes allele frequencies, increases inbreeding, and decreases genetic diversity is an inverse function of the effective population size (N_e) and thus exerts its greatest influence in the small populations of greatest conservation concern. The N_e of a real population is equivalent to the number of individuals in a theoretical, ideal population having the same magnitude of random genetic drift (Hartl & Clark 1997). In general, a number of factors are known to affect N_e , with most resulting in it being less than the standing number of reproductive individuals within a population. These factors include historical variation in population size, unequal numbers of males and females, and skewed distributions in family size (Crow & Kimura 1970). To our knowledge, however, little empirical data are available concerning the effects of clonal structure on N_e in plant species reproducing both sexually and asexually. In such species, if the reproductive success of genetically distinct clones (genets) is proportional to the number of its clonal subunits (ramets), then N_e will be maximized when clone sizes are small and will decrease as they become increasingly skewed. In self-compatible plants, the spatial clustering of clonal ramets, coupled with limited pollen dispersal, may further increase random genetic drift by promoting inbreeding via selfing among ramets of the same genet. Clonal structure thus has important implications for the conservation of small populations, where its effects on N_e and hence on rates of random genetic drift, levels of inbreeding, and standing genetic diversity are likely to have the most negative consequences for population fitness.

Orchidaceae is one of the largest families of flowering plants, with the estimated number of orchid species comprising up to 10% of all angiosperms (Dressler 1981). Many species exhibit both sexual and vegetative reproduction, consist of small, spatially isolated populations, and may be particularly susceptible to the effects of random genetic drift. Based on herbarium records and field surveys, the numbers of individuals of many terrestrial orchids, in particular, have rapidly decreased as a result of mass collection by plant sellers and enthusiasts and

habitat destruction and fragmentation caused by urban development (Alphonso 1975; Borromeo 1975; Pradhan 1975; Bowles et al. 1992; Wong & Sun 1999; Chung & Chung 2000). Thus, information on the demographic and genetic structure of surviving wild orchid populations is essential for formulating comprehensive plans for their conservation (Falk & Holsinger 1991; Hamrick & Godt 1996; Wallace 2002).

The terrestrial orchid *Cremastra appendiculata* (D. Don) Makino is a good model species for investigating the effects of clonal spread on random genetic drift and the relevance of these processes for conservation. This species reproduces both sexually and vegetatively and is rare throughout much of its range in eastern Asia. We investigated six small, isolated populations of *C. appendiculata* in a large (180 ha), undisturbed landscape on Oenaro Island, located off of the southeastern coast of South Korea. For each population we quantified the statistical significance and spatial scale of clonal spread with spatial autocorrelation analysis of ramets and genets for the populations. We then used this information to calculate three demographic estimators of N_e for each population. The first of these estimators assumes the absence of vegetative reproduction and equates N_e to the number of ecologically apparent individuals (ramets) within a population. This measure overestimates N_e but has the logistical benefit of being estimable from the direct observation of plants in the field. The second estimator equates N_e to the number of genets as determined by the number of distinguishable multilocus allozyme genotypes with a population. This approach takes into account the number of clones but not variation in relative fertility attributable to differences in clone size. The third estimator of N_e incorporates information on both the number of genets and variation in the number of ramets per genet, assuming that the relative fertility of genets is directly related to clone size (number of ramets per genet). Because these estimators differ in their assumptions about vegetative reproduction, they illustrate the extent to which cryptic clonal structure can result in the underestimation of random genetic drift and its potential impacts on population fitness. Together, our analyses provide a detailed, landscape-level picture of the consequences of clonal structure for N_e and random

genetic drift in individual *C. appendiculata* populations, with clear implications for the conservation of terrestrial orchids and other taxa possessing similar demographics and life-history traits.

Methods

Plant Species and Populations

Cremastra appendiculata has a geographical distribution ranging from Japan, southern Korea, China, and Taiwan to the Himalayas (Kitamura et al. 1986) and is extremely rare in southern Korea (M. Y. C. & M. G. C., personal observations). *Cremastra appendiculata* grows on rocky areas and in early summer normally produces a single leaf from a newly formed corm connected by a rhizome (about 2 cm long) to the corm made in the previous year. Corms are normally shallowly rooted and persist for several seasons, potentially giving rise to clonal spread via the growth of multiple shoots from the same genetic individual. Leaves are present from July through April and begin to wither as the 30- to 50-cm-tall inflorescence develops and flowers in May to June in southern Korea. Greenish brown flowers (20–50 per inflorescence) are visited by three species of bees and two species of syrphid flies, and among them only queens of the long-tongued bumblebee (*Bombus diversus diversus*) are likely to be effective pollinators (Sugiura 1996). Although flowers of *C. appendiculata* are self-compatible (successful fruit set occurs as a result of both artificial intra- and interinflorescence crosses and fruits develop from self-pollinated flowers; Chung & Chung, 2003), fruit set in natural habitats is low (mean fruit set per inflorescence = 1.3–2.0%; Sugiura 1996; Chung & Chung 2003). This suggests resource-limited fruit set or pollinator limitation (queens of *B. d. diversus* occur at low frequency) coupled with structural or phenological mechanisms limiting self-fertilization. Typical of orchids, fruits (2.0–2.5 cm long) contain large numbers of minute seeds.

Landscape-level variation in topography and the frequency and size of canopy gaps in the oak-maple (*Quercus-Acer*) overstory, in addition to other factors, may be important sources of variation in clonal structure. To encompass this variation, we established a study site containing six populations of *C. appendiculata*. This 180-ha (1000 × 1800 m) study site was located on Oenaro Island in Province Jollanam on the southern coast of Korea. This area is a part of Dadohaesang National Park and has been well preserved; there has been no apparent human interference for centuries at the site. We mapped and sampled leaf material from all observed adult-sized individuals ($n = 204$) in the six populations at the site, whether flowering or not. Subadult individuals were uncommon and not sampled for genetic analysis. We considered as nonflowering individuals with smaller leaf and

corm size than flowering adults and subadults. Hence, the sampled shoots represent all individuals potentially contributing to the effective size of each population. These populations formed two distinct spatial clusters (Fig. 1): (1) LOPA (100 × 150 m, elevation 280–320 m above sea level, $n = 139$ reproductive individuals) was on southeast-facing hillsides, where three populations were encountered (LOPA-1, LOPA-2, and LOPA-3) and (2) LOPB (120 × 160 m, elevation 270–310 m above sea level, $n = 65$) was also on southeast-facing hillsides and consisted of three populations (LOPB-1, LOPB-2, and LOPB-3). No individuals were found outside of these two regions of the 180-ha study area.

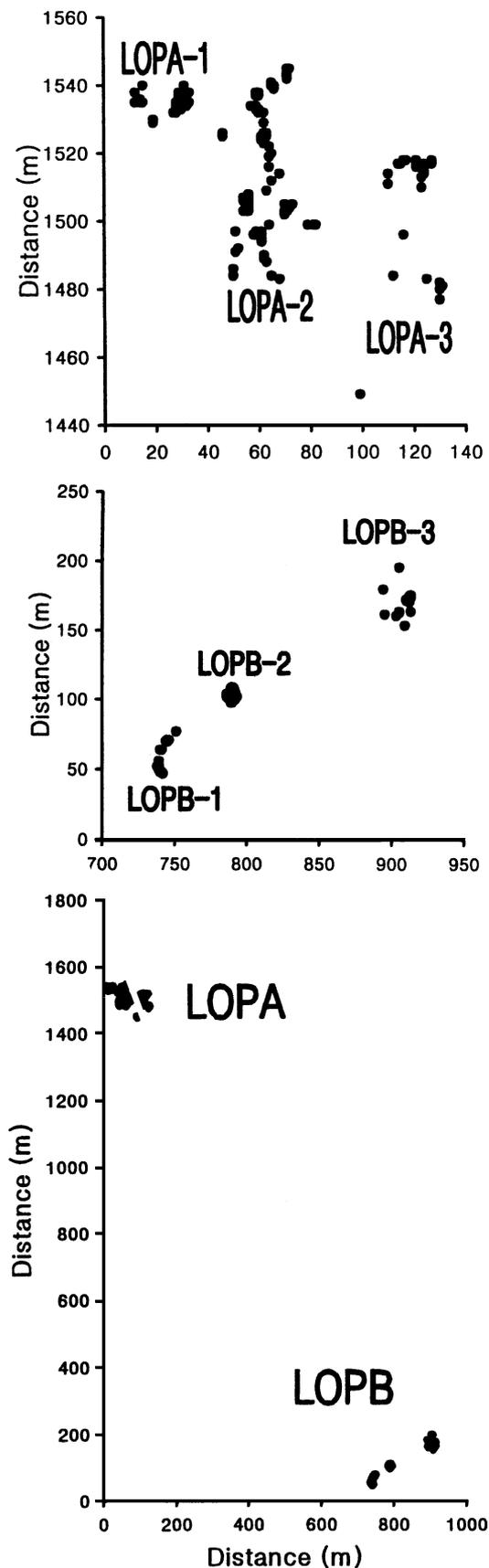
Electrophoretic Procedures

We kept all sampled leaf material on ice until it could be transported to the laboratory of M.G.C., where it was stored at 4° C until protein extraction. For extraction we finely cut and then crushed leaf samples with a mortar and pestle in a phosphate-polyvinylpyrrolidone extraction buffer (Mitton et al. 1979). Enzyme extracts were absorbed onto 4 × 6 mm wicks cut from Whatman 3MM chromatography paper, which were then stored at –70° C until needed. We determined levels and distribution of allozyme variation with horizontal gel electrophoresis. Starch gels (11%) were stained for 13 enzyme systems, which resolved 18 putative loci. We used a modification (Haufler 1985) of Soltis et al.'s (1983) system 6 to resolve alcohol dehydrogenase (*Adh*), diaphorase (*Dia-1*, *Dia-2*, *Dia-3*), fluorescent esterase (*Fe*), leucine aminopeptidase (*Lap*), malic enzyme (*Me*), phosphoglucosomerase (*Pgi-1*, *Pgi-2*), and phosphoglucosomutase (*Pgm-1*, *Pgm-2*). A modification (Chung & Kang 1994) of the morpholine citrate buffer system (pH 6.3) of Clayton and Tretiak (1972) was used to resolve formate dehydrogenase (*Fdb*), fructose-1, 6-diphosphatase (*Fl,6*), isocitrate dehydrogenase (*Idb*), malate dehydrogenase (*Mdb-1*, *Mdb-2*), 6-phosphogluconate dehydrogenase (*6Pgd*), and shikimate dehydrogenase (*Skdh*). Stain recipes were from Soltis et al. (1983), except diaphorase (Cheliak & Pitel 1984) and formate dehydrogenase (Wendel & Weeden 1989). We inferred the genetic basis of enzyme banding patterns from observed segregation patterns in light of typical subunit structure and subcellular compartmentalization (Weeden & Wendel 1989; Wendel & Weeden 1989). Putative loci were designated sequentially, with the most anodally migrating isozyme designated as 1, the next most as 2, and so forth. Likewise, alleles were designated sequentially, with the most anodally migrating allele designated as a.

Statistical Analyses

MEASURES OF CLONAL DIVERSITY

Because *C. appendiculata* is both sexually and vegetatively reproducing, for the purpose of estimating N_e it



is important to determine whether shoots with identical marker genotypes are clones (Berg & Hamrick 1994; Chung & Epperson 1999). The rigorous testing of clonal structure requires showing that (1) the available genetic markers have good statistical power to discriminate clonal genotypes from identical sexually produced genotypes, (2) putative clones are observed sharing the same multilocus marker genotype, and (3) putative clonal genotypes are spatially clustered as expected for growth via vegetative spread. The discriminating power of our markers was measured for each population as $1 - P_G$, where P_G —the probability that two random, sexually produced genotypes will be identical—was calculated as the product over loci of observed genotypic frequencies of genets assuming linkage equilibrium (Berg & Hamrick 1994). Because power was high for all populations (>0.95), we identified putative clonal ramets by inspection of the genotypic data.

To evaluate the spatial distribution of putative clones, we calculated spatial autocorrelation statistics (Sokal & Oden 1978) for the total number of “unlike” joins among multilocus genotypes (e.g., Chung & Epperson 1999). We calculated standard normal deviate (SND) with the program JCSP (B. K. Epperson, Michigan State University, East Lansing). An SND has an asymptotically standard normal distribution under the null hypothesis of random dispersion. An SND of <-1.96 indicates a significant deficit ($p < 0.05$) of pairs of unlike genotypes (and an excess of like genotypes) separated by a given range of Euclidean distances (Epperson 1993). Hence, significant negative values at short-distance intervals are indicative of real clonal structure, not a lack of power of the allozyme data to distinguish genotypes. Because of the small sizes of individual populations, analysis was conducted separately with LOPA and LOPB.

CLONAL EFFECTS ON GENETIC STRUCTURE

We measured the mean level of inbreeding within and genetic differentiation among populations with Wright's (1965) F_{IS} and F_{ST} , respectively, estimated over polymorphic loci according to the method of Weir and Cockerham (1984). In this analysis we did not nest populations within LOPA and LOPB because pairwise estimates of F_{ST} were not greater for comparisons between than within these two areas. To access clonal effects on genetic structure, we obtained estimates of F_{IS} and F_{ST} for both total

*Figure 1. Locations of six local populations of *Cremastra appendiculata* on Oenaro Island, Korea. Sites LOPA-1, LOPA-2, and LOPA-3 were on southeast-facing hillsides in LOPA (100 × 150 m, n = 139); LOPB-1, LOPB-2, and LOPB-3 were also on southeast-facing hillsides in LOPB (120 × 160 m, n = 65).*

samples (r) and genets (g) with 95% confidence intervals constructed over 1500 bootstrap replicates. These calculations were made with the program FSTAT (Goudet 2000). We also calculated F_{IS} separately for each population, both for total samples ($F_{IS(r)}$) and genets ($F_{IS(g)}$), with 95% bootstrap confidence intervals (1000 replicates) constructed using the program GDA (Lewis & Zaykin 2001).

In a related analysis, we used spatial autocorrelation techniques to quantify the effects of clonal structure on the spatial patterning of genetic variation within *C. appendiculata* populations. Following the methods of Kalisz et al. (2001), we plotted the mean coancestry (f_{ij}) between pairs of individuals i and j within each population as a function of the distance interval between them. We used 5-m lags (given the relatively small number of individuals within each population) with right-hand binning and constructed 95% and 99% confidence limits about the null hypothesis of no genetic structure ($f_{ij} = 0$) at each distance interval with randomization procedures (Kalisz et al. 2001). We explicitly evaluated the effects of clonal structure by conducting the spatial autocorrelation analyses on population samples containing all shoots versus restricted data sets compared with a single ramet per genet so as to exclude clones. In this latter case, the x, y coordinates of each ramet were placed at the genet's center of mass. To increase our power to reject the null hypothesis, we averaged coancestry estimates at each distance over populations with confidence limits adjusted accordingly. These averages excluded coancestry (i.e., F_{ST}) attributable to variation in allele frequency among populations (or, more precisely, the correlation of alleles within populations).

CLONAL EFFECTS ON N_e AND RANDOM GENETIC DRIFT

We obtained three estimates of effective population size. The $N_{e(r)}$ and $N_{e(g)}$ are the number of ramets and genets per population, respectively, and are equivalent when all shoots are genetically distinguishable. The third estimate, $N_{e(c)}$, takes into account both the number of genets and the variation in ramet number per genet. As a basis for estimating $N_{e(c)}$, we used $(4N - 2)/(V_k + 2)$ (Eq. 7.6.2.13 in Crow & Kimura 1970), where N is the number of distinct genets (here $N_{e(g)}$) and V_k is the variance in the number of gametes (k) contributed by individual genets to the next generation. Assuming random mating, constant population size, and binomial variation in the number of gametes contributed by individual ramets, V_k will be an increasing function of the number of ramets per genet.

Although the number of gametes contributed per genet may be an accelerating or decelerating positive function of the number of ramets per genet, because the exact form of this relationship is not known, we assumed a simple linear relationship as a reasonable approximation. Given these assumptions and the observed data on genet number and ramet number per genet, we used a Monte

Carlo simulation to simulate variation in the reproduction of ramets and genets within populations, from which we calculated V_k over genets and then $N_{e(c)}$ (using the equation above).

For each one of the three LOPA and three LOPB populations and for the total population, we generated 1000 simulated populations. For each set of simulated populations, we obtained a mean $N_{e(c)}$ and determined 95% confidence limits from the 25th and 976th of the 1000 ordered simulation estimates. We also calculated the relative evenness of ramet distributions within each population as $N_{e(c)}/N_{e(g)}$ (Menken et al. 1995), which is at its maximum value of one when variation in ramet number across genets is zero (i.e., equal numbers of ramets within genets).

The age structure of a *Cremastra* population also influences its effective size. Although our formulation of $N_{e(c)}$ is based on nonoverlapping generations, the dependency of N_e on V_k is more general (Hill 1979). Here we used observed variation in clone size to determine variation in reproductive success among genets because information on lifetime reproductive success in *C. appendiculata* is unavailable. When possible, however, detailed demographic data on lifetime reproductive success should be used to calculate V_k for populations with overlapping generations (Lande & Barrowclough 1987; Nunney & Elam 1994).

To determine the degree to which clonal structure potentially increases random genetic drift, we calculated for each population the expected per-generation increase in the population inbreeding coefficient, $\Delta F = 1/2N_{ef}$, using $N_{e(r)}$, $N_{e(g)}$, and $N_{e(c)}$ as estimators of the inbreeding effective population size, N_{ef} . Random genetic drift can also be expressed in terms of the per-generation change in allele frequency (variance effective size) or loss of heterozygosity (eigenvalue effective size). Because these three measures of random genetic drift are very similar under most circumstances, we focused on the inbreeding effective size because this concept is the most widely used (Hartl & Clark 1997).

Results

Measures of Clonal Diversity

Of the 18 loci examined, 7 were polymorphic (*Dia-1*, *Fdb*, *Fe*, *Mdb-2*, *6Pgd*, *Pgm-1*, and *Pgm-2*). Allozyme variation was moderate and homogeneous across populations, with a mean percentage of polymorphic loci within populations of 34% and a mean number of alleles per locus of 1.40. Genetic diversity (expected heterozygosity) calculated from both total population samples and samples excluding identical multilocus genotypes (and hence clones) was 0.122.

Table 1. Summary of clonal diversity and estimates of Wright's F_{IS} for six local populations of *Cremastra appendiculata*.^a

No. of ramets per genet	No. of genets per population ^b						Total _(g)
	LOPA-1	LOPA-2	LOPA-3	LOPB-1	LOPB-2	LOPB-3	
1	3	17	12	7	3	7	49
2	2	8	4	2	2	2	20
3	3	5	1	2	3	1	15
4	1	0	1	0	1	1	4
5	0	3	0	0	0	0	3
6	0	1	0	0	0	0	1
10	0	0	0	0	1	0	1
11	0	1	0	0	0	0	1
12	1	0	0	0	0	0	1
$N_{e(r)}$	32	80	27	17	30	18	204
$N_{e(g)}$	10	35	18	11	10	11	95
$N_{e(c)}$	6.39	23.92	16.18	10.67	7.36	9.84	60.95
$N_{e(c)}$ 95% CI	4.33-8.98	18.36-29.89	11.38-21.64	7.10-14.62	5.01-10.30	6.47-13.58	50.55-72.19
P_G	0.0417	0.0081	0.0195	0.0207	0.0189	0.0461	0.0258
$F_{IS(r)}$	-0.445	0.025	-0.172	-0.103	-0.280	-0.354	-0.159 ^c
$F_{IS(r)}$ 95% CI	-0.705-0.042	-0.125-0.157	-0.384 (-0.021)	-0.397-0.188	-0.538-0.078	0.567-0.178	-0.261 (-0.026)
$F_{IS(g)}$	-0.258	0.049	-0.084	-0.051	-0.182	-0.215	-0.070 ^c
$F_{IS(g)}$ 95% CI	-0.478-0.072	-0.063-0.157	-0.347-0.207	-0.275-0.252	-0.389-0.168	-0.529-0.036	-0.194-0.065

^aAbbreviations: $N_{e(r)}$, number of ramets (shoots); $N_{e(g)}$, number of genets (genotypes); $N_{e(c)}$, demographic effective population size; P_G , probability of randomly drawing two identical genotypes; F_{IS} , fixation index; 95% CI, 95% bootstrap confidence intervals. Subscripts r and g refer to total samples (including clonal ramets) and samples restricted to genets, respectively.

^bRefer to Methods for descriptions of populations.

^cMean across the six populations.

Given the available marker variation, our power to discriminate clonal genotypes from sexually produced genotypes identical by chance alone was >0.95 for each population (mean $P_G = 0.0258$; Table 1). Given this power, we treated ramets sharing the same genotype as putative clones, finding that, within populations, 30–70% of genets formed clones consisting of two or more ramets (Table 1). Join-count statistics revealed a statistically significant deficit of joins between unlike multilocus genotypes only at distances of <12 m within LOPA and <4 m within LOPB (Fig. 2). Together, these results indicate that the positive spatial clustering of identical genotypes is consistent with the expectations of clonal structure.

Clonal Effects on Genetic Structure

Wright's F_{IS} calculated for our population samples including clonal structure was significantly less than zero (mean $F_{IS(r)} = -0.159$), with fixation indices for individual populations ranging from -0.445 for LOPA-1 to 0.025 for LOPA-2 (Table 1). When population data were constrained to genets (excluding clonal structure), F_{IS} was negative but not significant (mean $F_{IS(g)} = -0.070$) and individual population fixation indices ranged from -0.258 for LOPA-1 to 0.049 for LOPA-2 but were not significantly different from zero for any population (Table 1). Estimates of Wright's F_{ST} calculated for data including and excluding clonal structure both indicated high and significant variation in allele frequency among populations (respectively: $F_{ST(r)}$

= 0.209, 95% CI 0.098, 0.283; $F_{ST(g)} = 0.189$, 95% CI 0.094, 0.261).

Spatial autocorrelation analyses indicate significant fine-scale genetic structure within populations of *C. appendiculata* (Fig. 3). When clonal ramets were included in the analysis, there was significant positive coancestry at

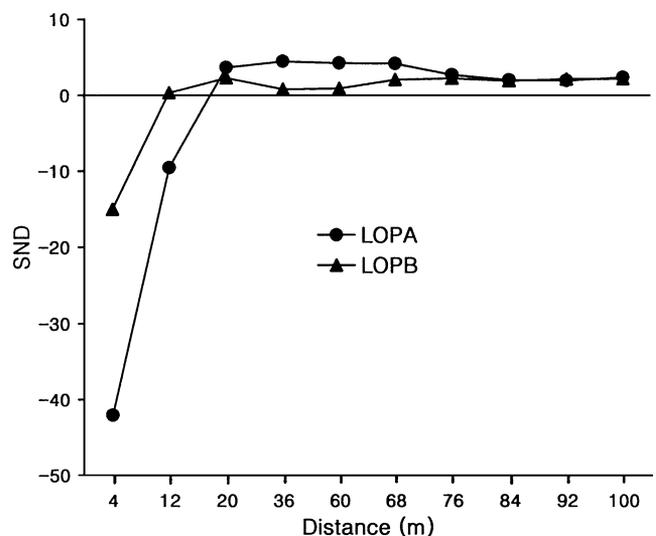


Figure 2. Correlograms for populations LOPA and LOPB of *Cremastra appendiculata* showing the relationships between pairs of the total number of unlike joins among multilocus genotypes (SND, standard normal deviate).

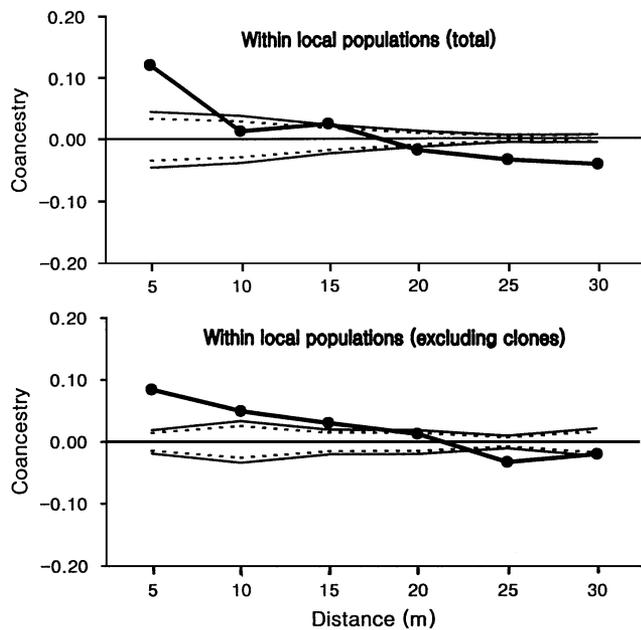


Figure 3. Spatial autocorrelation analyses of coancestry (f_{ij}) averaged over six populations of *C. appendiculata*. Mean f_{ij} versus interplant distance for total samples (including clonal ramets) and for sample genets (excluding clones). The solid and dashed lines represent the upper and lower 99% and 95% confidence envelopes, respectively, around the null hypothesis of $f_{ij} = 0$.

0–15 m, with the highest mean coancestry ($f_{ij} = 0.121$) at 5 m, the shortest evaluated distance interval. When clonal ramets were excluded from the analysis, mean coancestry remained significant at 0–15 m, decreasing only to $f_{ij} = 0.084$ at 5 m. Together, these results indicate the positive spatial aggregation of clones (clonal structure) and the presence of related genets (family structure) within *C. appendiculata* populations.

Clonal Effects on N_e and Random Genetic Drift

The observed number of adult shoots or ramets ranged from 17 to 80 per population, with a total of 204 ramets over the entire study area (Table 1). Genetic analysis identified 10–35 genets per population (95 in total), with one-third to two-thirds of these genets consisting of multiple ramets (2–12 ramets per clone). Averaged over populations, the effective population size estimated from the number of genets (mean $N_{e(g)} = 15.8$) was less than half that estimated from the number of ramets (mean $N_{e(r)} = 34$). Taking into account the number of genets and variation in genet size further reduced our estimates of the effective population size, with $N_{e(c)}$ ranging from 6.39 to 23.92 and a mean of 12.39 (Table 1). The $N_{e(c)}$ estimates were significantly less than the observed number of ramets ($N_{e(r)}$) in all six populations and less than the

observed number of genets ($N_{e(g)}$) in LOPA1 and LOPA2. For the total population, $N_{e(g)} = 60.95$ was significantly less than the observed number of both ramets and genets (Table 1).

Discussion

Impacts of Clonal Structure on N_e

We documented substantial clonal structure within populations of the rare terrestrial orchid *C. appendiculata*. Given that the theoretical relationship between effective population size (N_e) and random genetic drift is nonlinear, how this clonal structure influences rates of random genetic drift depends on the value of N_e . For $N_e > 50$, relatively large differences among populations in N_e translate into relatively small differences in random genetic drift and its impacts on population fitness (as measured by the per-generation increase in inbreeding; Fig. 4). As N_e becomes increasingly small, however, as in the case of our *C. appendiculata* populations, even relatively small differences in N_e are expected to have large effects on random genetic drift. Consequently, when the true N_e and estimates of it (e.g., $N_{e(r)}$, $N_{e(g)}$, and $N_{e(c)}$) are large, the choice of estimator has a negligible effect on the inferred per-generation rate of random genetic drift. In contrast, as the true N_e decreases, it becomes increasingly important that the estimator be accurate. Hence, a good understanding of factors influencing N_e is especially important in small populations that are likely to be of concern for conservation.

Using genetic markers to quantify ecologically cryptic clonal structure in *C. appendiculata* populations

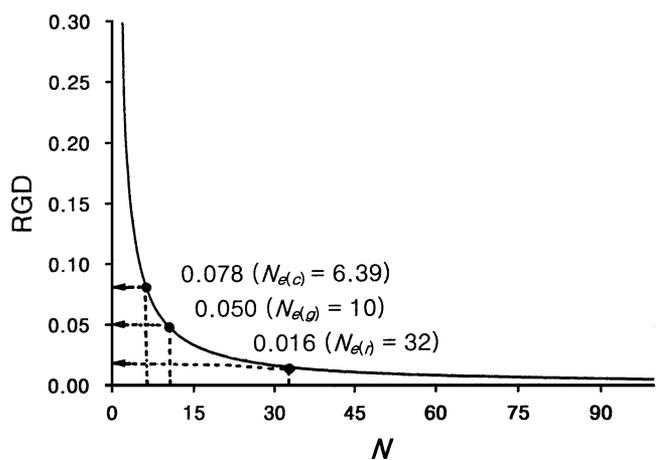


Figure 4. The effect of effective population size on random genetic drift (RGD), measured as the expected per-generation increase in inbreeding. Indicated values of RGD are for population LOPA-1, where $N_{e(r)} = 32$, $N_{e(g)} = 10$, and $N_{e(c)} = 6.39$.

resulted in up to a fivefold reduction in the estimated N_e ($N_{e(c)}$ vs. $N_{e(r)}$; Table 1). In LOPA-1, for example, $N_{e(r)} = 32$ ramets and $N_{e(g)} = 10$ genets, and when variation in clone size (number of ramets per genet) was taken into account, we obtained $N_{e(c)} = 6.39$. This reduction in the estimated N_e was associated with an approximately fivefold increase in the expected rate of random genetic drift (Fig. 4)—in particular, a per-generation increase in inbreeding from 0.02 to 0.08. To put this in a biological and conservation perspective, this latter level of inbreeding is intermediate to that expected for the offspring of first cousin mating (0.0625) and half-sib mating (0.125), a degree of consanguinity that may generate significant inbreeding depression in historically outbreeding species (e.g., Nason & Ellstrand 1995). Thus, failure to take into account clonal structure in species with vegetative reproduction could lead one to substantially underestimate the level of inbreeding and its short- and long-term impacts on individual fitness and population survival.

The quantification of clonal structure also has important implications for predicting the rate at which genetic diversity has been and will be lost due to random genetic drift. In finite populations, the proportional reduction in expected heterozygosity (H_e) after t generations of random genetic drift is given by the recursion $(1 - 1/(2N_e))^t$. We used this simple recursion with estimators of N_e that do ($N_{e(c)}$) and do not ($N_{e(r)}$) take into account the clonal structure observed in *C. appendiculata* populations to determine the proportional reduction in H_e for 45 generations (Fig. 5). Figure 5 illustrates how rapidly gene diversity can be lost at small N_e (Fig. 5a) and how this problem is further exacerbated by the presence of clonal structure (Fig. 5b). In LOPA-1, for example, if the true N_e is equal to the number of ramets ($N_{e(r)} = 32$), a 50% loss of heterozygosity is expected in 44 generations under random genetic drift. In contrast, if $N_e = N_{e(c)} = 6.39$, and thus reflects the effects of clone number and size, a similar reduction in genetic diversity is expected in only nine generations. Clearly, in small populations clonal structure can strongly accelerate the rate at which potentially adaptive genetic variation is lost as a result of random genetic drift. Accordingly, such structure should be accounted for in management strategies aimed at the preservation of endangered plant populations.

Reconciling Small N_e with Observed Patterns of Genetic Variation

The six populations of *C. appendiculata* we studied consisted of relatively few individual ramets ($N_{e(r)} = 17$ to 80) and even fewer genets ($N_{e(g)} = 10$ to 35). Moreover, the estimated effective sizes were even smaller ($N_{e(c)} = 6.39$ to 23.92) when we assumed that variation among genets was a linear function of genet size (number of ramets). Given the spatial isolation and, by any measure, small effective sizes of these populations, we would expect them

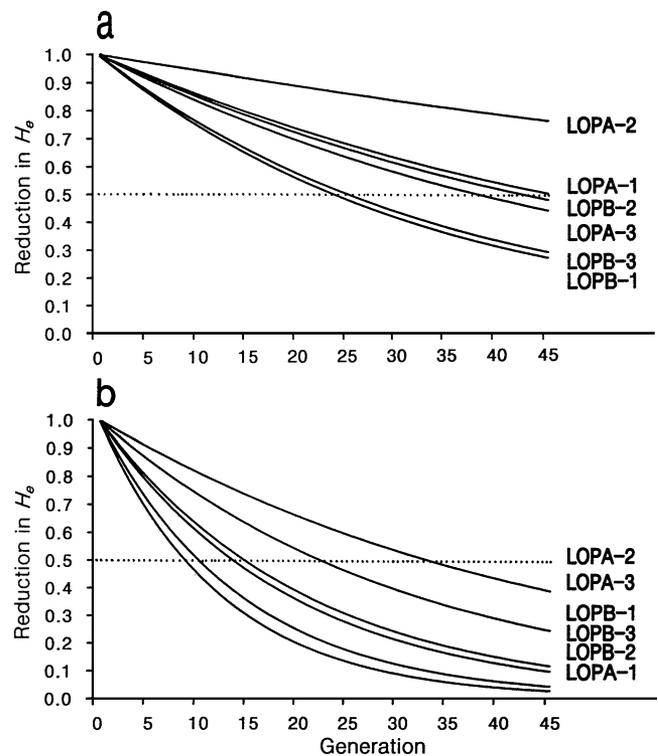


Figure 5. Proportional reduction in expected heterozygosity (H_e) of *C. appendiculata* populations (LOPA-1, LOPA-2, LOPA-3, LOPB-1, LOPB-2, and LOPB-3) under random genetic drift, given effective population sizes estimated from (a) the number of ramets ($N_{e(r)}$) and (b) the number of genets and variation in genet size ($N_{e(c)}$).

to experience dramatic losses of genetic diversity and elevated levels of inbreeding. However, *C. appendiculata* populations exhibit the moderate genetic diversity and low levels of inbreeding typical of other long-lived perennial herbs (Hamrick & Godt 1989). Why? These data imply that the historical N_e is larger than the current N_e given the demographic and clonal structure. Thus, in answering this question we must pose solutions that explicitly relate to the value of N_e .

Several processes may account for the maintenance of gene diversity within *C. appendiculata* populations. One possibility is that, rather than consisting of six spatially and genetically isolated populations, *C. appendiculata* functions more as a single, larger population integrated by high rates of gene flow. Although the pollen- and seed-dispersal biology of *C. appendiculata* is not well understood, our analyses revealed a high level of allele frequency differentiation ($F_{ST(g)} = 0.189$) among patch populations separated by approximately 40–2000 m. Moreover, the low $F_{IS(g)}$ within populations indicates the lack of significant Wahlund effect at this spatial scale. As a result, the observed fine-scale genetic structure (when adjusted for clonal structure; Fig. 3) indicates localized seed

dispersal and not isolation by distance among cryptic population subdivisions. Similar genetic evidence of localized seed dispersal has been found for three other terrestrial orchids, *C. tentaculata* (Peakall & Beattie 1996), *Cymbidium goeringii* (Chung et al. 1998), and *Cephalanthera longibracteata* (Chung et al. 2004). Given these observations, the data do not support the hypothesis of a large N_e maintained by landscape-level, interpopulation gene flow in *C. appendiculata*.

Two alternative hypotheses for the maintenance of genetic diversity assume a historical perspective. One hypothesis is that the set of populations we examined was only recently established by genetically diverse founders and that there has not been sufficient time (generations) for this initial diversity to be substantially eroded by random genetic drift. The other is that the effective sizes of the study populations are much smaller today than they were in the recent past. Although there is no evidence of human-mediated disturbance in our study area on Oenaro Island, perhaps LOPA and LOPB previously comprised more continuously distributed populations of large N_e . In either case, given the small effective sizes of *C. appendiculata* populations, two observations support the conclusion that the underlying demographic events implied by these hypotheses must have occurred within the past few generations. First, as noted above, we expect genetic diversity to decline rapidly under random genetic drift. In contrast, the standing genetic diversity was relatively high. Second, we expect deficits of low-frequency alleles to accompany bottlenecks in N_e because of founder events or crashes in population size, but we found no evidence of genetic bottlenecks (data not shown) when we used the methods of Cornuet and Luikart (1996) and Piry et al. (1999).

Although it is not the focus of this research, future studies may reveal the expression of inbreeding depression at multiple life stages in *C. appendiculata*, including pollination, fertilization, fruit and seed maturation, and recruitment. However, although strong selection against inbreeding should decrease the per-generation increase in inbreeding measured in adult plants, it cannot eliminate inbreeding in small, finite populations. Indeed, at their current effective population sizes, future *C. appendiculata* generations are likely to experience substantial declines in population fitness resulting from inbreeding depression and losses of adaptive genetic variation.

Conservation Implications

The long-term survival of isolated populations of fewer than several hundred individuals is threatened by a variety of stochastic demographic and genetic processes with negative consequences for population fitness (e.g., Soulé 1987; Les et al. 1991; Menges 1991; Byers & Meagher 1992). As a result primarily of human-mediated factors, many terrestrial orchid species currently consist of small,

isolated populations of concern for conservation. Our results demonstrate that failure to take into account clonal structure in populations of the size of *C. appendiculata* will often lead to overestimates of N_e and, hence, underestimates of the implications of genetic drift for individual and population fitness. Because the demographic and life-history characteristics of *C. appendiculata* are representative of those of many terrestrial orchids, efforts to account for clonal structure should be particularly relevant to their conservation. Current conservation strategies for many terrestrial orchid species have in fact been formulated without the benefit of information on clonal structure. If this structure is extensive and ecologically cryptic, as in *C. appendiculata*, then effective population sizes may be smaller and the rates and impacts of random genetic drift greater than anticipated under current management plans.

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