Fine-scale Genetic Structure among Genetic Individuals of the Clone-Forming Monotypic Genus *Echinosophora koreensis* (Fabaceae)

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● *Background and Aims* For rare endemics or endangered plant species that reproduce both sexually and vegetatively it is critical to understand the extent of clonality because assessment of clonal extent and distribution has important ecological and evolutionary consequences with conservation implications. A survey was undertaken to understand clonal effects on fine-scale genetic structure (FSGS) in two populations (one from a disturbed and the other from an undisturbed locality) of *Echinosophora koreensis*, an endangered small shrub belonging to a monotypic genus in central Korea that reproduces both sexually and vegetatively via rhizomes.

● *Methods* Using inter-simple sequence repeats (ISSRs) as genetic markers, the spatial distribution of individuals was evaluated using Ripley's $L(d)$-statistics and quantified the spatial scale of clonal spread and spatial distribution of ISSR genotypes using spatial autocorrelation analysis techniques (join-count statistics and kinship coefficient, F_{ij}) for total samples and samples excluding clones.

● *Key Results* A high degree of differentiation between populations was observed ($\Phi_{ST(g)} = 0.184$, $P < 0.001$). Ripley's $L(d)$-statistics revealed a near random distribution of individuals in a disturbed population, whereas significant aggregation of individuals was found in an undisturbed site. The join-count statistics revealed that most clones significantly aggregate at $\leqslant$6-m interplant distance. The Sp statistic reflecting patterns of correlograms revealed a strong pattern of FSGS for all four data sets ($Sp = 0.072$–0.154), but these patterns were not significantly different from each other. At small interplant distances ($\leqslant$2 m), however, jackknifed 95 % CIs revealed that the total samples exhibited significantly higher F_{ij} values than the same samples excluding clones.

● *Conclusion* The strong FSGS from genets is consistent with two biological and ecological traits of *E. koreensis*: bee-pollination and limited seed dispersal. Furthermore, potential clone mates over repeated generations would contribute to the observed high F_{ij} values among genets at short distance. To ensure long-term *ex situ* genetic variability of the endangered *E. koreensis*, individuals located at distances of 10−12 m should be collected across entire populations of *E. koreensis*.

Key words: Clonal structure, conservation, *Echinosophora koreensis*, monotypic genus, Fabaceae, fine-scale genetic structure, genets, ISSRs, sampling strategies.

INTRODUCTION

Clonal reproduction is a common fact in many plant species. Mechanisms of clonal reproduction include vegetative spread, production of bulbils, and apomictic seeds. For rare, endemic and/or endangered plant species that reproduce both sexually and vegetatively, it is important to characterize clonality because clonal structure could affect overall population fitness. In self-compatible plant species, for example, it has been suggested that increasing levels of clonal structure will increase the likelihood of self-fertilization (inbreeding) by pollination among different ramets of the same genets (i.e. geitonogamy; Handel, 1985). Thus, the spatial distribution of clonal *vs*. that of sexual reproduction generally has differing effects on the levels of biparental inbreeding and apparent selfing rate, produced via mating by proximity through limited pollen dispersal (Peakall and Beattie, 1991; Eckert and Barrett, 1993). In outcrossing plant species, extensive clonality could reduce reproductive potential, overall population fitness, and

effective population size, probably due to decreased probability of mating with different genets. Thus, knowledge of clonal structure within populations is crucial for understanding evolutionary processes in clonal plants.

Spatial statistical methods provide powerful tools for measuring the structure of genetic diversity within populations of target plants. Fine-scale spatial genetic structure (FSGS) can be quantified using spatial autocorrelation analysis to investigate population genetic processes (Sokal and Oden, 1978; Epperson, 1990; Heywood, 1991). When FSGS is analysed within populations of clonal plants, it is important to separate the number of genets (genetic individuals established via seeds) from the total samples including clonal ramets because inferences for micro-evolutionary processes and for conservation purposes from the pattern and magnitude of FSGS (such as the extent of pollen and seed dispersal, degree of inbreeding and genetic patch sizes) must be addressed from the number of genetic individuals (Chung and Epperson, 1999; Reusch *et al*., 1999; Hämmerli and Reusch, 2003; Alberto *et al*., 2005; Chung *et al*., 2005a, b). Therefore, it is essential to analyse fine-scale clonal structure using genetic markers *a priori* to understand

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better micro-evolutionary processes in clonal plant species. This point is relatively understudied among plant population geneticists, though there have been many studies on clonal diversity and structure within plant populations (e.g. Lynch *et al.*, 1998; Sydes and Peakall, 1998; Suzuki *et al.*, 1999; Burke *et al.*, 2000; Rossetto *et al.*, 2004; Torimaru and Tomaru, 2005; many references therein).

Inter simple sequence repeats (ISSR) are useful markers for estimating genetic variation within populations (Wolfe and Liston, 1998). Recent studies on clonal diversity of plant species have shown the great discriminating power of ISSR makers for separating genets from their clonal ramets within populations (Zietkiewicz *et al.*, 1994; Esselman *et al.*, 1999; Li and Ge, 2001; Xie *et al.*, 2005).

In plant populations, limited seed dispersal generates, in the same way as clonality, spatial clustering of full- and half-sib cohorts (Hamrick and Nason, 1996; Kalisz *et al.*, 2001). For clonal plants, if pollen dispersal is also spatially restricted within populations, then this sibling structure would further enhance biparental inbreeding or selfing via mating by proximity (Wright, 1969). Under this scenario, it is predicted that, repeated over generations, genetically distinct individuals would form a significant spatial clustering, and high levels of genetic similarity (strong spatial autocorrelation) between pairs of genets would exist over short distances.

Echinosophora koreensis was chosen as a study species for two reasons: (1) it reproduces both sexually and vegetatively; (2) it is endemic to Korea and belongs to a monotypic genus restricted to only a few localities in central Korea and may be at risk of extinction. To test the genetic prediction described above, two isolated populations of *E. koreensis* in central Korea were selected. The area (13 200 m^2) covering the two populations has been designated as a Natural Monument of Korea (#372) since 1992. Then spatial distribution of individuals and their genotypes at the ramet and genet levels were quantified using ISSRs as genetic markers. To do this, spatial statistics [Ripley's $L(d)$-statistics] and spatial autocorrelation methods (join-count statistic and kinship coefficient, F_{ij}) were used. With the information obtained on clonal and genetic structure, sampling strategies for *ex situ* conservation were provided.

MATERIALS AND METHODS

Study species

Echinosophora koreensis (Nakai) Nakai (Fabaceae) is a small shrub (<1 m tall) which belongs to a monotypic genus endemic to Korea (Park, 1973; Yim, 1993). In South Korea, populations of the species are known only in a few locations in Yanggu-gun and Injae-gun, in Kangwon-do Province (K. Huh, pers. comm.). Populations in Yanggu-gun and Injae-gun are considered to be at the southern limit of its distribution in Korea. Due to its rarity, the species is listed among the 58 endangered plant species proposed by the Ministry of Environment to be targeted for conservation in South Korea (http://www.me.go.kr). According to herbarium records, the species was also known in a few locations in North Korea (Myongsan, Pyangannam-do Province, and Bukchung, Hamgyeongnam-do Province). At present, there are no available samples from North Korea, and North Korean populations, if they still exist, have no protection measures in place. Based on available data concerning the total number of mature individuals (population size) for South Korea at the regional level (Gärdenfors *et al.*, 2001), *E. koreensis* can be classified as endangered (EN) following criteria of the IUCN Red List C2a(i) (IUCN, 2001).

Echinosophora koreensis is a pioneer species that can potentially invade open, sunny forest gaps, and disturbed areas (Yim, 1993). The natural habitats are xeric, consisting of small granite gravel, where shoots are interconnected by rhizomes (a 180-cm-long rhizome was identified in one population), indicating that the species propagates both sexually and vegetatively. Ten to twenty yellow flowers (3–5 cm long) per inflorescence bloom in May and are frequently visited by the bumblebee *Bombus diversus diversus* (M. Y. Chung and M. G. Chung, personal observation). The species was found to be self-compatible under greenhouse conditions (M. Y. Chung and M. G. Chung, unpubl. res.). Pods (approx. 7 cm long) ripen from July to September and contain two or three seeds. There are apparently no specialized mechanisms of seed dispersal.

Studied sites and sampling procedure

Two study sites were selected in the centre of each of two populations in Yanggu-gun, where 286 visually identified shoots with fruits were mapped and leaf samples were collected. The first population (hereafter referred to HAN, 30 × 40 m in area, approx. 135 m a.s.l., $N_{(r)} = 150$) is located on a north-east-facing hillside in Hanjeon-ri (Yanggu-eup), occupied by dead trunks of *Pinus densiflora* remaining from a fire in the area in December 2000. The second population (hereafter referred to YIM, 30 × 40 m in area, approx. 210 m a.s.l., $N_{(r)} = 136$), 2·8 km south of HAN, is located on a north-facing hillside in Imdang-ri (Yanggu-eup) under a low-density old stand of *Pinus densiflora*. In YIM, other shrubs were cleared by government officials in an attempt to give more sun to *E. koreensis*. One leaf was collected from each flowering shoot of *E. koreensis* and stored at 4 °C until DNA was extracted.

DNA isolation and polymerase chain reaction (PCR) amplification

For ISSR–PCR, a total of 24 ISSR primers [University of British Columbia (UBC), Canada] were screened using four representatives from each of the two populations. Among them, six primers that gave clear polymorphisms were used in this study (Table 1). Total genomic DNA was extracted from fresh foliage by QIAGEN Plant Mini Kit. The amount of DNA was directly quantified with DynaQuantTM 200 (HOEFER Phamacia Biotech Inc.). The total volume of reaction mixtures for PCR amplification was 20 µL, which contained 5 ng of template DNA, 0·2 mM of each dNTP, 0·0025 % of BSA (Boehringer Manheim, Germany), 520 µL of 1·5 µM primers (UBC, Canada), 1·2 µL of

Table 1. *Inter-simple sequence repeat (ISSR) primers used in this study*

Primer no.	Sequence
807	AGAGAGAGAGAGAGAGT
808	AGAGAGAGAGAGAGAGC
810	GAGAGAGAGAGAGAGAT
824	TCTCTCTCTCTCTCTCG
829	TGTGTGTGTGTGTGTGC
830	TGTGTGTGTGTGTGTGG

Table 2. *Summary of clonal and genetic diversity estimates of two populations of* Echinosophora koreensis

No. of ramets per genet	No. of genets per population	
	HAN	YIM
1	21	21
2	17	11
3	8	9
4	4	6
5	3	1
6	3	1
7	2	0
8	1	1
10	0	1
13	0	1
$N_{(r)}$	150	136
$N_{(g)}$	59	52
P_G	3.16×10^{-18}	4.76×10^{-18}
$\%P_{(r)}$	80.5 %	87.8 %
$\%P_{(g)}$	80.5 %	87.8 %
$h_{(r)}$	0.181	0.226
$h_{(g)}$	0.164	0.208

$N_{(r)}$, number of ramets (shoots); $N_{(g)}$, number of genets (genotypes); P_G, probability that two random, sexually produced multilocus genotypes will be identical; $\%P$, percentage of polymorphic loci; h, genetic diversity. Subscripts (r) and (g) refer to total samples (including clonal ramets) and samples restricted to genets, respectively.

$25\,\text{mM}$ MgCl$_2$ and 0.6 units of *Taq* DNA polymerase (Advanced Biotechnique, UK). Amplifications were performed in a PTC-200 thermocycler (MJ Research, USA) using a period of 5 min for one cycle for initial denaturation at 94 °C, followed by 45 cycles of 30 s of denaturation at 94 °C, 30 s annealing at 50 °C (for UBC #807 and #810) and 52 °C (for UBC #808, #824, #829 and #830), 1 min of extension at 72 °C, and a final extension step of 10 min at 72 °C. Amplification products of PCRs were fractioned on 1.5 % agarose gel in 1× TBE (Tris–boric acid–ethylenediamine tetraacetic acid) buffer at pH 8.0, stained with ethidium bromide, and photographed over a UV transilluminator. DNA size was calculated by comparing samples with a 100-bp DNA ladder (GIPCO BRL, USA) which was loaded in two separate lanes on the same gel for electrophoresis. To score ISSR fragments, it was assumed that each marker fragment or band represented the phenotype at a single biallelic distinct locus (Williams *et al.*, 1990; Dawson *et al.*, 1995). Smeared and weak bands of ISSRs were excluded. The majority of plant-primer combinations were run more than once to ensure reproducibility. ISSR fragments were scored as presence (1) and absence (0) of putative homologous bands, and were then transformed into a binary matrix.

Data analysis

Clonal structure. Since *E. koreensis* reproduces both sexually and vegetatively, it is important to determine whether shoots with identical marker genotypes are clones (Berg and Hamrick, 1994; Chung and Epperson, 1999; Chung *et al.*, 2004; Torimaru and Tomaru, 2005). To carry out these analyses, the available genetic markers must have enough statistical power to discriminate clonal genotypes from identical sexually produced genotypes. In order to determine the probability (P_G) that two random, sexually produced multilocus genotypes will be identical under the assumption of random mating, the following calculation was performed (e.g. Sydes and Peakall, 1998; Rossetto *et al.*, 2004): $P_G = \Pi\, p_i$, where p_i is the frequency of presence or absence of each band i in the multilocus dominant ISSR genotype. The discriminating power of the markers used in the present study was measured for each population as $1 - P_G$. Since power was high and similar for both populations ($1 - P_G \approx 1.0$, Table 2), identical multilocus genotypes were considered as clones. Hereafter, reference is made to subscripts (r) and (g) for total samples [$N_{(r)}$] and samples restricted to genets [$N_{(g)}$],

in order to distinguish between total samples and samples excluding clones for analysing levels of genetic diversity and FSGS.

Join-count statistic. It is expected that putative clonal genotypes would be spatially clustered as expected for spreading via rhizomes. To test this hypohesis, spatial autocorrelation statistics (Sokal and Oden, 1978) were calculated for the total number of 'unlike' joins among multilocus genotypes (e.g. Chung and Epperson, 1999; Chung *et al.*, 2004). Standard normal deviates (SND) were calculated using 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 < -1.96 indicates a significant ($P < 0.05$) deficit of pairs of unlike (and excess of like joins) genotypes separated by a given range of Euclidean distances (Epperson, 1993). Hence, significant negative values at short distance intervals are indicative of clonal structure.

Spatial distribution of individuals. To assess the spatial distribution of individuals, Ripley's $L(d)$-statistics (Ripley, 1976, 1977) were used. Ripley's $L(d)$ is calculated from the number of point pairs within concentric circles of increasing radii (d) around each plant. Since the use of circles with a radius greater than half the shortest plot side introduces excessive bias due to edge effects, radial distances of 1–15 m with 1-m lag were selected for each population (e.g. Parker *et al.*, 1997; Burke *et al.*, 2000; Cruse-Sanders and Hamrick, 2004; Ng *et al.*, 2004). Values of $L(d) = 0$, $L(d) > 0$ and $L(d) < 0$ indicate spatial randomness, spatial clustering and spatial repulsion (hyperdispersal), respectively, up to distance d. Confidence intervals

(CIs; 95 %) about the null hypothesis of spatial randomness were estimated by Monte Carlo simulation (199 replicates) with a value of $L(d)$ outside of this envelope judged to be a significant departure from the null hypothesis. A univariate analysis was also conducted for samples that excluded clonal ramets [$N_{(g)}$]. In this latter case the x and y co-ordinates of each ramet were placed at the genet's centre of mass. All calculations and simulations were performed using a program developed by P. Aldrich (Smithsonian Institution, National Museum of Natural History, USA) and E. Berg (Kenai National Wildlife Refuge, USA).

ISSR diversity. To estimate levels of genetic diversity, the percentage of polymorphic loci (P) was calculated. In order to estimate Nei's genetic diversity (h) in populations (Nei, 1973), an allozyme analysis was conducted on the same populations and inbreeding coefficients ($F_{IS} = 0.090$ and 0.120 at HAN and YIM, respectively: M. Y. Chung and M. G. Chung, unpubl. res.) were calculated. The estimates of h were calculated on the basis of F_{IS} values using the program POPGENE version 1.31 (Yeh *et al.*, 1999) for total samples and samples excluding clones. The program GenAlEx (Peakall and Smouse, 2001) was used to assess the partitioning of the genetic variance within and between populations by an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) in which analogues of F-statistics (so-called Φ statistics) are extracted. Estimation of these genetic parameters was based both on total samples and samples excluding clones.

Fine-scale genetic structure. To quantify the effects of clonal structure on the FSGS within *E. koreensis* populations, FSGS were analysed for both total samples and samples excluding clonal ramets. The program SPAGeDi (Hardy and Vekemans, 2002) was used to analyse FSGS, since (*a*) this program provides 95 % CIs of the slope (*blog*) of each correlogram and jackknifed means of *blog* of correlograms and their standard errors across loci, and (*b*) it allowed FSGS on the binary dominant data set (presence or absence of a certain fragment) to be analysed. The analysis of the pairwise kinship coefficients F_{ij}, (Loiselle *et al.*, 1995; Kalisz *et al.*, 2001) with dominant markers in diploids was outlined in Hardy (2003). The estimation of F_{ij} with ISSR markers (i.e. dominant markers) requires that F_{IS} is known (Hardy, 2003). Therefore, F_{IS} estimated on the basis of allozyme markers described above was used.

To visualize FSGS, values of F_{ij} located within the distance classes (2-m intervals) were averaged and plotted against the distance. To assess statistical significance of the average F_{ij}, each F_{ij} value was compared with 95 % CIs generated under the null hypothesis of no spatial genetic structure ($F_{ij} = 0$). Sample multilocus binary data were drawn at random with replacement and assigned to occupied map locations within the study population. Re-sampling was repeated 999 times, and the observed F_{ij} values represented the 1000th statistic, for each distance class. The 95 % CIs were constructed as the interval from the 25th to the 976th ordered permutation estimates. Values of F_{ij} that fall above or below the 95 % bootstrapped CIs are interpreted as showing significantly greater or lesser genetic structure ($P < 0.05$), respectively, than expected at random.

Under an isolation-by-distance model, Rousset (1997, 2000) showed that the probability of identity in state between two neutral genes decreases approximately linearly with the logarithm of spatial distance in two dimensions at a rate proportional to $1/D\sigma^2$, where D is the effective population density and σ^2 is half the average squared axial parent–offspring distance. Similarly, pairwise kinship coefficients, $F(r_{ij})$, between individuals i and j separated by distance r decrease linearly with the logarithm of distance r in a two-dimensional space (Vekemans and Hardy, 2004). Following the Vekemans and Hardy's method, $F(r_{ij})$ values were regressed on the logarithm of spatial distance r_{ij} to obtain the regression slope (*blog*) for each data set (total samples and excluding clones in HAN and YIM, respectively). To test for FSGS (i.e. isolation by distance), each *blog* was evaluated using a Mantel test with 1000 permutations under null hypothesis (*blog* = 0). Since *blog* depends to some extent on sampling scale used and it is negative, the Sp statistic reflecting the rate of decrease of pairwise kinship with distance was estimated (Vekemans and Hardy, 2004). The statistic allowed within-population FSGS patterns within the present study to be compared among each other and to patterns observed in other taxa (e.g. Alberto *et al.*, 2005; reviewed in Vekemans and Hardy, 2004). The Sp statistic is equal to $-blog/[1 - F_{(i=1)}]$ (Vekemans and Hardy, 2004), where $F_{(i=1)}$ is the mean F_{ij} at the smallest distance interval [hence $F_{(i=1)} = F_{(2\,m)}$]. To determine whether a significant difference of the Sp values exists between four comparisons, t-tests were conducted, assuming that the distribution of the Sp values per each locus was approximately normal.

Finally, to test whether mean values of F_{ij} at $\leqslant 2$ m between total samples and samples excluding clones are significantly different each other, the approximation of a 95 % CI (± 1.96 times the standard error) was used. All analyses, including estimation of jackknifed standard errors, were conducted using the program SPAGeDi.

RESULTS

Clonal structure

Given the available marker variation, the power to discriminate clonal genotypes from sexually produced genotypes identical by chance alone was $1 - P_G \approx 1.0$ for each population ($P_G = 4.76 \times 10^{-18}$ to 3.16×10^{-18}; Table 2). Given this power, ramets sharing the same genotype were treated as putative clones, finding that within populations 85–86 % of genets formed clones consisting of two or more ramets (Table 2). Clones ranged in size from one to eight ramets at HAN and one to 13 ramets at YIM. However, the distribution of clone sizes was not significantly different between populations (contingency χ^2-test: $\chi^2 = 7.33$, d.f. = 9, $P = 0.603$). Join-count statistics revealed a statistically significant deficit of joins between unlike multilocus genotypes (i.e. excesses of pairs of identical genotypes) compared with random distribution expectations, only at distances of $0\,m < d < 2\,m$ (standard normal deviate test statistic, SND = -51.3 for HAN and -38.9 for YIM) and for $2\,m < d < 6\,m$ (SND = -19.4 for HAN and -11.3 for
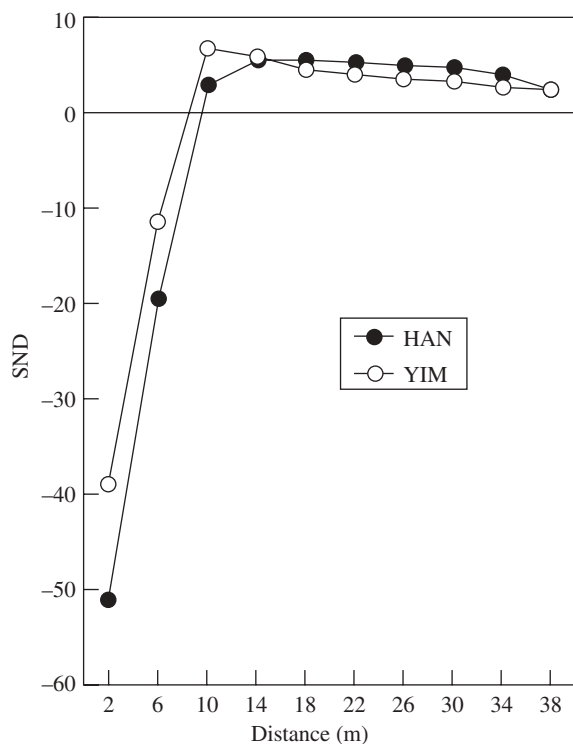
FIG. 1. Correlograms for populations HAN and YIM of *Echinosophora koreensis* showing the relationships between pairs of the total number of unlike joins among multilocus genotypes (SND, standard normal deviate).

YIM), but not at greater distances within the two populations (Fig. 1). Together, these results indicate the positive spatial clustering of identical multilocus genotypes, consistent with the expectations of clonal structure.

Spatial distribution of individuals

Visual inspection of the spatial arrangement of plants in HAN suggested a less clumped distribution, whereas a more clumped distribution of shoots was observed in YIM. Ripley's $L(d)$-statistics indicated a somewhat patchy distribution of shoots at <6 m and a hyperdispersal distribution beyond this distance to 15 m in HAN. However, when clones are excluded, a near random distribution of genets is evident at 2–13 m (Fig. 2). In contrast to HAN, significant aggregation both of total shoots and genets was found at YIM for all distance intervals (Fig. 2).

ISSR diversity

Of the 41 putative ISSR loci examined, 36 were polymorphic (100 % criterion) across two populations (%$P = 87.8$ %). Genetic diversity calculated from both total population samples and samples excluding identical multilocus genotypes (and hence clones) was slightly higher for YIM [$h_{(r)} = 0.226$ at YIM *vs*. 0.181 at HAN] but with similar estimates between total samples and those excluding clones (Table 2). AMOVA revealed moderate levels of genetic variability among individuals within populations and significant and high degrees of differentiation between
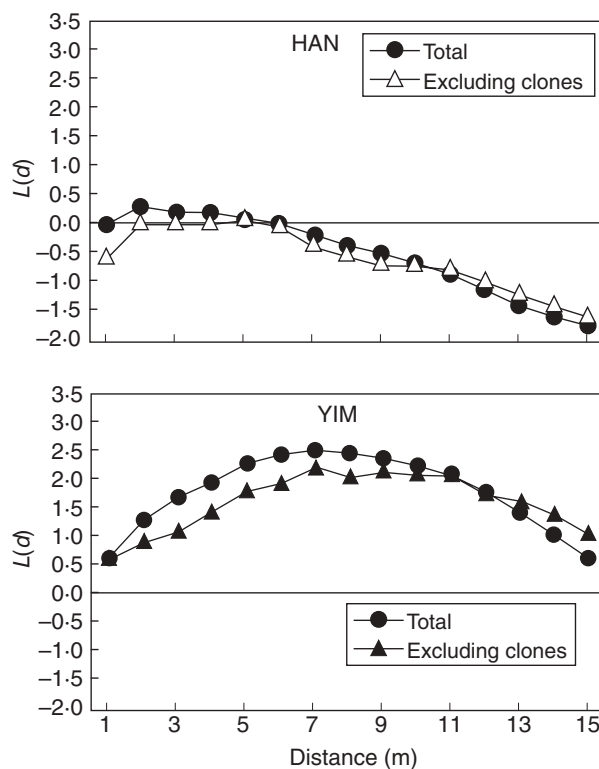

FIG. 2. Results of Ripley's $L(d)$-statistics, observed $L(d)$ estimates for univariate Ripley analysis in two populations (HAN and YIM) of *Echinosophora koreensis* for total samples (including clonal ramets; circles) and for sample genets (excluding clones; triangles). Estimates that depart significantly from the null hypothesis [$L(d) = 0$] are indicated by closed symbols.

populations ($\Phi_{ST(r)} = 0.226$ for total samples, $P < 0.001$; $\Phi_{ST(g)} = 0.184$ for samples excluding clones, $P < 0.001$).

Fine-scale genetic structure

Thirty-three (HAN) and 36 (YIM) loci were chosen for spatial autocorrelation analysis according to the criteria described in Materials and methods. Overall slopes ($blog$) of all four correlograms were significantly different from the null hypothesis of no spatial genetic structure ($blog = 0$): $blog_{(r)}$ of -0.114 (95 % CI = -0.005, 0.004) for total samples and $blog_{(g)}$ of -0.076 (95 % CI = -0.012, 0.010) for genets at HAN; $blog_{(r)}$ of -0.142 (95 % CI = -0.006, 0.004) for total samples and $blog_{(g)}$ of -0.080 (95 % CI = -0.012, 0.010) for genets at YIM (Fig. 3).

Sp values revealed strong patterns of FSGS for total samples and they were very close between the two populations [$Sp = 0.148 \pm 0.040$ (s.e.) at HAN; 0.154 $\pm$ 0.040 at YIM]. Values decreased about 2-fold were found in samples excluding clones (0.072 $\pm$ 0.033 at HAN; 0.093 $\pm$ 0.034 at YIM). These patterns, however, were not significantly different from each other.

In contrast, the magnitude of F_{ij} at ≤2 m differed significantly between total samples and samples excluding clones at HAN (mean: 0.372, 95 % CI = 0.310, 0.437 for total; mean: 0.160, 95 % CI = 0.085, 0.237 for genets)
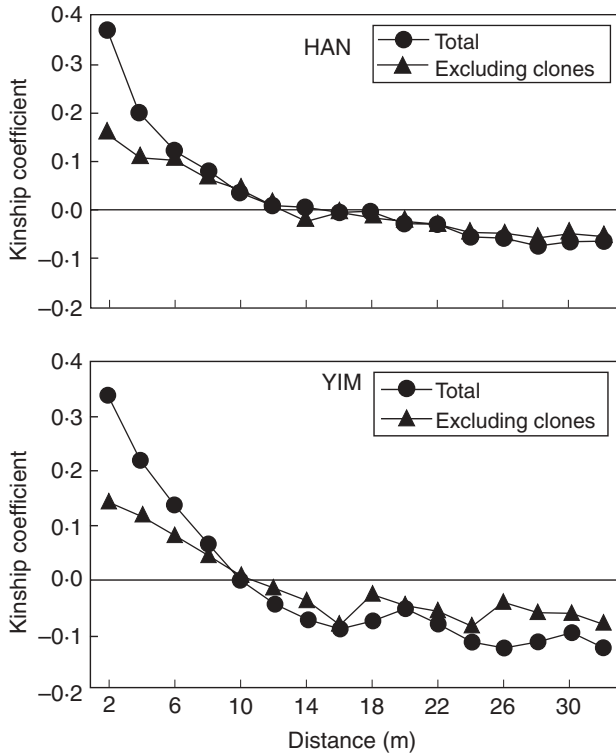
FIG. 3. Correlograms of kinship coefficients (F_{ij}) in two populations (HAN and YIM) of *Echinosophora koreensis* for total samples (including clonal ramets; circles) and for genets (excluding clones; triangles). Estimates that depart significantly from the null hypothesis ($F_{ij} = 0$) are indicated by closed symbols.

and YIM (mean: 0·339, 95 % CI = 0·262, 0·424 for total; mean: 0·142, 95 % CI = 0·042, 0·241 for genets). Differences between populations for total samples and genets, however, were not significantly different at this spatial scale.

The distance at which the mean F_{ij} value first intercepts the *x* co-ordinate may represent the length of a genetic patch size (Sokal and Wartenberg, 1983; Smouse and Peakall, 1999; Epperson, 2003; Escudero *et al.*, 2003). Although *x* is sensitive to the spatial scale of sampling and is not a characteristic of populations (Fenster *et al.*, 2003; Vekemans and Hardy, 2004), it can be used to compare similarly sampled populations. The average intercept was similar between total samples and samples excluding clones in both populations. The approximate patch size is 10–12 m (Fig. 3).

DISCUSSION

Levels of genetic diversity

Moderate levels of genetic diversity found within the total samples were similar to those found in samples excluding clones. This indicates that clones do not significantly affect levels of genetic diversity within populations of *Echinosophora koreensis*. Mean genetic diversity within populations was $h_{(g)} = 0·204$, and the percentage of polymorphic loci was $\%P = 87·8\ \%$. These values are higher than

those found in other endemic species (Li and Ge, 2001; Ge *et al.*, 2005; Sheng *et al.*, 2005; Xie *et al.*, 2005). This comparison suggests that the marginal populations of *E. koreensis* are not genetically depauperate. However, genetic differentiation between the two populations of *E. koreensis* was high for total samples [$\Phi_{ST(r)} = 0·226$]. This differentiation was somewhat lower when considering only genets [$\Phi_{ST(g)}$], with estimates decreasing to 0·184. This level of differentiation may reflect occasional pollen flow through bumblebees but restricted seed dispersal between populations separated by 2·8 km.

Spatial distribution and fine-scale genetic structure

Join-count statistics revealed that most clones aggregate significantly at ≤6-m interplant distance, which reflects the spatial distribution of ramets at short distances. Significant aggregation of both total shoots and genets was found at YIM. In contrast to this, HAN showed a near random distribution of genets but a slight aggregation of shoots at <6 m. This suggests different population histories and/or ecological conditions between the two sites. At YIM many distinct patches grow in gaps under high density of *Pinus densiflora* overstorey. At HAN, in contrast, only a few *P. densiflora* grew before a recent fire. The entire area surrounding and including HAN had been disturbed by domesticated animals (e.g. cows and goats) before designation of Natural Monument in 1980s. The relatively open habitat of HAN provides suitable light for *E. koreensis* individuals. Clones may, therefore, be more likely to proliferate at HAN.

Significant evidence of FSGS was detected in two populations both at the ramet and genet levels, and a similar pattern of correlograms between treatments (ramets *vs.* genets) and populations was found. The pronounced FSGS found in samples excluding clones (genets) of two populations ($Sp = 0·072$ and $0·093$) suggests restricted dispersal of *E. koreensis*. The *Sp* values estimated here are within the range of those reported in predominantly selfing herbaceous species, particularly in leguminous species ($Sp = 0·055–0·263$; Zoro Bi *et al.*, 1997; Bonnin *et al.*, 2001; reviewed in Vekemans and Hardy, 2004). The finding that HAN showed a near random distribution of genets but a significantly positive autocorrelation of genotypes at <10 m may need explanation. Disturbance (e.g. fire) and open habitats at HAN may have encouraged colonization and establishment at the HAN site with the initially established genets spreading in various directions over <6 m distance. With a relatively high density of clonal ramets, many of these may have died randomly via thinning, resulting in a weak aggregation of shoots and a near random distribution of genets. Genotypes, however, still showed significant clumping in short-distance intervals as seen in a population of *Pinus clausa* in Florida, USA (Parker *et al.*, 2001).

FSGS within populations is primarily determined by the effects of limited seed and pollen dispersal, isolation in small demographic patches, and microhabitat requirements (Wright, 1943; Linhart *et al.*, 1981; Slatkin and Arter, 1991; Hamrick and Nason, 1996; McCauley, 1997;

Parker *et al.*, 2001; Chung *et al.*, 2003). Among these factors, probably the most widely studied influence on patterns of FSGS is seed dispersal (Hamrick and Nason, 1996). If, at the scale of investigation, seed dispersal is localized, it will result in spatial clustering of genetically related individuals (full-sibs, half-sibs, first cousins, etc.) and will result in the development of significant FSGS (Wright, 1943; Sokal and Wartenberg, 1983; Barbujani, 1987). In *E. koreensis* no specialized seed or pod dispersal mechanism is known. Thus, limited seed dispersal may be a primary factor responsible for the observed spatial genetic structuring in this species. Nonetheless, the strong (magnitude of F_{ij} at $\leqslant 2$ m) FSGS observed in genets may also be related to limited pollen dispersal. The observed pollinators of *E. koreensis* in the HAN populations were bumblebees which exhibited a large proportion of short flight distances [2·54 $\pm$ 1·34 (s.d.) m, $n = 54$] within patches (M. Y. Chung and M. G. Chung, unpubl. res.), though bumblebees may travel between patches as well [a leptokurtic dispersal of pollen; Roubik (1989)]. The effect of the mating system on the level of genetic structure (i.e. F_{IS}) will primarily influence between-individual kinship (F_{ij}; Cockerham, 1969) values of the correlograms at the shortest distances, because at short distances F_{ij} values are highly correlated with inbreeding (Crow and Kimura, 1970; Hardy and Vekemans, 1999). Under random mating, F_{ij} between individuals is a measure of the inbreeding coefficient of their hypothetical offspring with expected values of 0·25 for full-sibs, 0·125 for half-sibs, and 0·0625 for first cousins (Crow and Kimura, 1970). Mean $F_{ij}(g)$ of 0·160 (HAN) and 0·142 (YIM) were estimated from the data set excluding clones at $\leqslant 2$-m interplant distance, which corresponds to F_{ij} values expected for half-sibs (Crow and Kimura, 1970). Considering this high level of relatedness, it is further suggested that, since clonal ramets aggregate at $\leqslant 6$ m, the potential of selfing via geitonogamy over generations in *E. koreensis* might lead to the high F_{ij} values observed among genets within a distance of 2 m.

To sum up, there were no statistically significant differences in the *Sp* values of the four treatments (total samples and excluding clones from each of two populations), although approx. 2-fold stronger patterns were detected in total samples. In addition, similar *x*-intercepts were also found when comparing total samples and samples excluding clones. Thus, clones appear to have little effect on patch size as inferred from the correlogram intercepts. However, at $\leqslant 2$-m interplant distance, the total samples exhibited significantly higher F_{ij} values than population samples excluding clones, primarily due to the aggregation of clonal ramets at $\leqslant 6$ m distance. This indicates that clones themselves enhance the *magnitude* but not *patterns* of spatial autocorrelation. In terms of evolutionary processes, the strong pattern of FSGS found in genets of *E. koreensis* would be due to limited pollen and seed dispersal, probably coupled with clonal effects on breeding structure.

Implications for conservation

Although local populations of *E. koreensis* currently maintain moderate levels of genetic diversity within populations, the two populations are at risk due to illegal collectors and high human frequentation. These activities could decrease $N_{(g)}$ in the two studied sites. Furthermore, only 45 flowering shoots were located in another isolated population in Wolmyeong-ri (M.Y.C. and M.G.C., personal observation), suggesting that $N_{(g)}$ in this population is small, and random genetic drift could be significant. Long-term genetic variability of *E. koreensis* can only be ensured by implementing appropriate conservation and management strategies. The distribution of genetic variation in space is the prime factor to take into account in the conservation and management of natural plant populations (McCue *et al.*, 1996). For example, sampling methods for seed stocks and decisions on the size of an area for population preservation may greatly benefit from this information. The estimation of the average intercept across alleles has important implications for *ex situ* and *in situ* conservation. Based on the correlogram intercepts of average F_{ij} in local populations of *E. koreensis*, individuals closer than 10–12 m tend to be quite similar genetically, representing 'pseudoreplication' in terms of genetic variability. To optimize sampling design and avoid pseudoreplication, only individuals at greater distances should be collected (Diniz-Filho and Telles, 2002), and they should be sampled from each location since the two populations are significantly differentiated.

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