

POPULATION STRUCTURE AND GENETIC DIVERSITY OF *BOTRYCHIUM PUMICOLA* (OPHIOGLOSSACEAE) BASED ON INTER-SIMPLE SEQUENCE REPEATS (ISSR)¹

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Species of *Botrychium* reproduce by spores that form subterranean gametophytes and a few, like *B. pumicola*, also reproduce asexually with subterranean sporophytic gemmae. The goal of this study was to examine the genetic diversity of *B. pumicola* populations and to better understand the role of gemmae. Ninety-nine individuals from three monitored populations were sampled. The technique of inter-simple sequence repeats (ISSR) produced 15 polymorphic loci and identified 71 ISSR genotypes. Sixteen of the ISSR genotypes were shared by more than one individual in a population, representing potential clones. Ten of the 16 shared genotypes were not limited to clusters of plants (groups of plants growing from the same point). The ten potential clones were disjunct (separated by other genotypes) and not in patches as might be expected for an underground propagule. There is a high probability that these shared genotypes arose from independent sexual events suggesting they were not clones. These results suggest that the long-distance dispersal of gemmae is at best a rare event.

Key words: *Botrychium*; gemmae; ISSR; Ophioglossaceae; population genetics.

The reproduction of Ophioglossaceae, including *Botrychium* Swartz, occurs underground. Species in this family of eusporangiate ferns produce a bisexual subterranean gametophyte that requires prolonged darkness and the formation of mycorrhizae to develop (Whittier, 1973). Underground, the flagellated sperm fertilizes an archegonium and a sporophyte develops. Cross-fertilization may be hindered by the need for two gametophytes to be close enough for the sperm of one individual to reach the egg of another (Tyron and Tyron, 1982). In another plant group with underground gametophytes, the Lycopodiaceae, self-fertilization appears to be rare (Soltis and Soltis, 1988). Wagner, Wagner, and Beitel (1985) provided theoretical arguments for possible outcrossing in species of *Botrychium*, including the common occurrence of allopolyploid hybrids. However isozyme studies suggest inbreeding within a species is the norm (McCauley, Whittier, and Reilly, 1985; Soltis and Soltis, 1986; Watano and Sahashi, 1992; Farrar, 1998).

In addition to reproduction by spores, some species of Ophioglossaceae have subterranean asexual reproduction. The genus *Ophioglossum* L. can reproduce by means of root buds (Cascio and Thomas, 1993), and some species of *Botrychium* reproduce asexually by achlorophyllous subterranean sporophytic gemmae (Farrar and Johnson-Groh, 1990).

Botrychium pumicola Coville reproduces by gemmae (Camacho, 1996). Of 25 observations of the belowground organs of *B. pumicola*, no gametophytes were found, only gemmae. This observation led to the hypothesis that gemmae are an important part of the life cycle for this species. The gemmae of *B. pumicola* are 0.4 mm wide and develop on the underground stem. The gemmae do not appear capable of easily dispersing away from the parent plant.

In Oregon, *Botrychium pumicola* occurs in Deschutes, Klamath, and Lake counties (Wagner and Wagner, 1993) and is one of Oregon's rarest ferns. This species is also reported from Mt. Shasta, California (W. H. Wagner, personal communication, University of Michigan). It occupies specialized habitats in subalpine communities and open frost pockets of lodgepole pine (*Pinus contorta* Loudon) forest at lower elevation montane sites. These habitats are typically sparsely vegetated raw pumice and pumice-rich soils. Population sizes range from 1 to 1500 or more plants (Hopkins and O'Neil, 1993).

Molecular markers have been widely used to characterize clones in plants (Sheffield, Wolf, and Haufler, 1989; Bayer, 1990; Parker and Hamrick, 1992; Smith, Bruhn, and Anderson, 1992; Parks and Werth, 1993; Neuhaus et al., 1993; Hsiao and Rieseberg, 1994; Stiller and Denton, 1995; Waycott, 1995; Ayres and Ryan, 1997; Montalvo et al., 1997). These molecular techniques represent the only reasonable way of distinguishing ramets from a genet, in a fragmenting clonal plant (Parks and Werth, 1993). Inter-simple sequence repeats (ISSR) within a species can be a highly variable region of DNA (Salmath et al., 1995). ISSR have the advantage over randomly amplified polymorphic DNA (RAPD) in that the primers are longer, allowing for more stringent annealing temperatures (Wolfe and Liston, 1998). These higher temperatures apparently provide a higher reproducibility of bands than in RAPD (Nagaoka and Ogihara, 1997; Wolfe, Xiang, and Kephart, 1998). Tsumura, Ohba, and Strauss (1996) found that most of their ISSR bands (96%) segregated according to Mendelian expectations. Our study used ISSR to examine three populations of *B. pumicola* in order to evaluate asexual reproduction in this species.

MATERIALS AND METHODS

Three mapped populations of *Botrychium pumicola* on the Deschutes National Forest were sampled during the summer of 1997 (Table 1). Ninety-nine individuals were sampled. Individuals were sampled by removing a portion of the leaf. These leaves were stored on ice and then at -20°C . DNA was extracted with a Qiagen (Chatsworth, California, USA) DNeasy plant extrac-

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TABLE 1. Location of *Botrychium pumicola* populations and the number of individuals sampled.

Site name	Latitude	Longitude	Oregon county	No. of plants 1992–1996	Individuals sampled	Environment
Paulina View	43°42'N	121°12'W	Deschutes	339	27	subalpine
Broken Top	44°04'N	121°41'W	Deschutes	493	49	subalpine
Katati-2	43°27'N	121°18'W	Lake	153	23	montane

tion kit. A subset of the samples was used to screen ISSR primers for polymorphic loci.

Each ISSR reaction was carried out in a total volume of 17 μ L, containing 8.5 μ L MasterAmp 2X PCR PreMix D and one unit of AmpliTherm polymerase (Epicentre, Madison, Wisconsin, USA), 5.5 μ L double deionized water, 1 μ L BSA (10 mg/mL), 1 μ L of primer (10 nmol/mL), and 10–20 ng of genomic DNA (1 μ L volume). Initial denaturation was carried out for 1 min at 94°C, followed by 34 cycles of 45 sec at 94°C, 30 sec at 50°C, 2 min 15 sec at 72°C, and a final 5 min extension at 72°C. ISSR primers were obtained from the University of British Columbia Biotechnology Laboratory. Polymerase chain reaction (PCR) was performed in an MJ-Research (Watertown, Massachusetts, USA) PTC-100 thermocycler. Products were analyzed on 1.2% agarose gels in 1X TBE buffer and stained with ethidium bromide. Band size was estimated from a 100 bp ladder (NEB, Beverly, Massachusetts, USA). Loci were named based on the primer and observed band size.

Data were scored as presence and absence of bands. Percentage polymorphic loci, allele frequencies, Nei's genetic diversity, measures of population differentiation, and Shannon's index of phenotypic diversity (King and Schaal, 1989) were computed with POPGENE 1.20 (Yeh et al., 1997). NTSYSpc 2.02 (Rohlf, 1997) was used to conduct a unweighted pair-group method using an arithmetic average (UPGMA) analysis using the Dice coefficient and a Mantel test, which examines the correlation between the matrix of genetic distance and spatial distance within a site. This test is a randomization procedure that compares the correlation between two matrices with the correlation between one of these and random permutations of the other. By use of allele frequencies, the probability that each genotype could arise independently was calculated following Parks and Werth (1993).

RESULTS

Twenty-two primers were evaluated for their ability to produce polymorphic bands (putative loci) with a subset of the *Botrychium pumicola* samples. Six of the primers were determined to produce interpretable and variable banding patterns (Table 2). For the 99 samples, these 6 primers produced 29 scorable bands, 15 of which were polymorphic. Four of these primers had a CT sequence repeat, producing 11 of the 15 bands. The polymorphic bands for each CT primer were of a unique size, suggesting that different loci were amplified.

The spatial relationships of the shared and unique genotypes in each population are shown in Figs. 1, 2, and 3. Sixteen

TABLE 2. Primers used in inter-simple sequence repeat (ISSR) analyses of *Botrychium pumicola* and size of the bands they produced. R = A, G; Y = C, T.

Primer name	Primer sequence	ISSR band sizes in base pairs
UBC-813	(CT) ₈ T	320, 390, 450, 485, 530, 600, 630
UBC-814	(CT) ₈ A	350, 450, 500, 520, 540, 610, 730
UBC-824	(TC) ₈ G	450, 560, 600, 740, 770, 860
UBC-845	(CT) ₈ RG	420, 480
UBC-840	(GA) ₈ YT	450, 500, 550, 590, 600, 650
UBC-848	(CA) ₈ RG	440

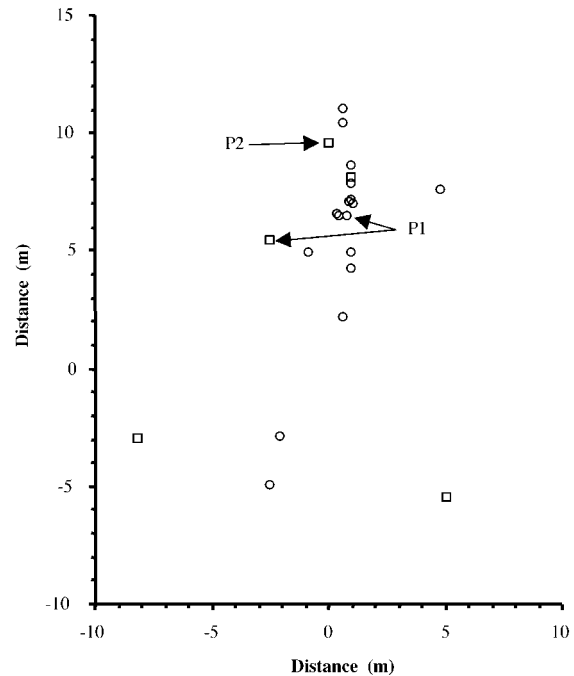


Fig. 1. Paulina View population of sampled specimens of *Botrychium pumicola*. Circles represent single plants and squares represent a cluster of two plants. Plants with shared genotypes P1 and P2 (Table 3) are designated with arrows. Only one of the plants in the cluster assigned to P1 has that genotype.

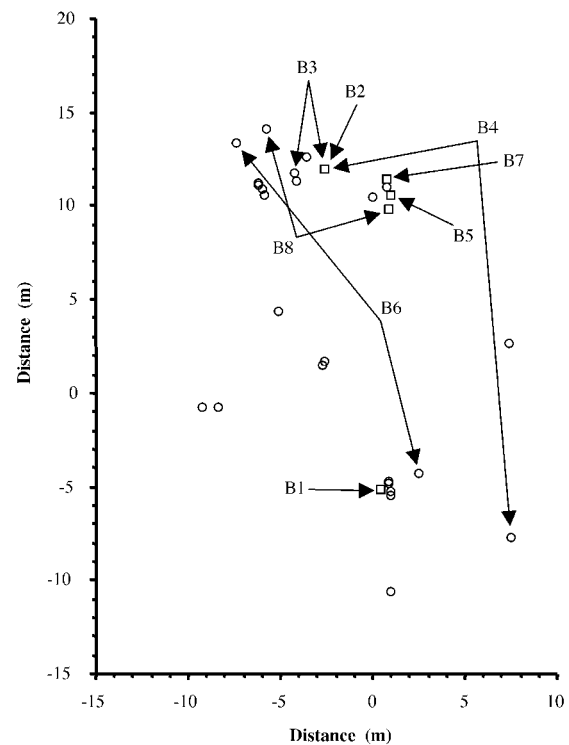


Fig. 2. Broken Top population of sampled specimens of *Botrychium pumicola*. Circles represent single plants and squares represent a cluster of plants. Plants with the shared genotypes B1 through B8 (Table 3) are designated with arrows. A cluster of three plants contains individuals with genotypes B2, B3, and B4. One of each of the B2 and B3 genotype plants is not mapped.

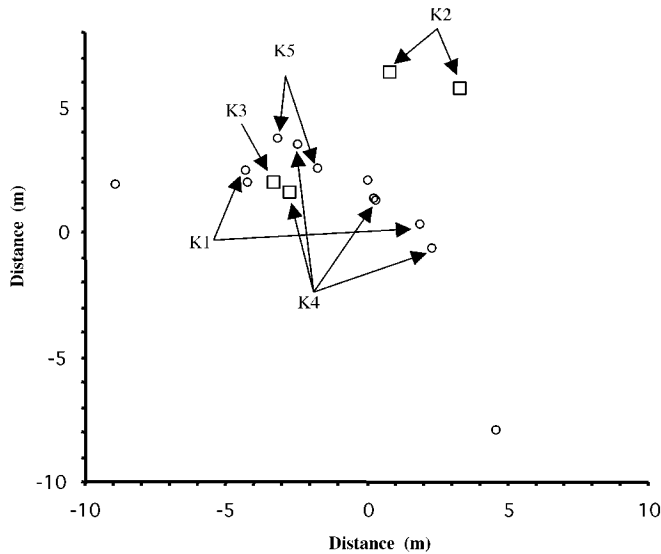


Fig. 3. Katati-2 population of sampled specimens of *Botrychium pumicola*. Circles represent single plants and squares represent a cluster of plants. Plants with the shared genotypes K1 through K5 (Table 3) are designated with arrows.

multilocus genotypes were shared by more than one plant within a population (Table 3). Two of these genotypes also had individuals from another population (not shown). Most shared genotypes were represented by only two plants (Table 3). The highest number of individuals sharing a genotype at a site was five. Two of these five were in a cluster, a group of plants growing from the same point. In nine of the 13 plant clusters sampled, the individuals in the cluster shared a genotype. It cannot be determined whether these identical plants in a cluster originated from the reproduction of gemmae or multiple intragametophyte self-fertilization events. Because of the difficulty of determining reproduction modes in a cluster, spatially separated plants within a population may be more appropriate for the detection of asexual reproduction in *Botrychium pumicola*. Such disjunct shared genotypes occurred in

TABLE 3. The shared genotypes, the distance between individuals, and the probability of a genotype occurring a second time by random mating. Genotypes are listed in order of probability scores, going from lowest to highest. Cluster = zero distance, NA = not available.

Genotypes	No. of individuals	Distances between plants (m)	Probability of second encounter
B7	2	cluster	0.007
B1	2	cluster	0.010
K3	2	cluster	0.015
B9	2	cluster	0.028
B5	2	cluster	0.079
B2	2	Na (not cluster)	0.098
K5	2	1.8	0.109
P1	2	3.5	0.186
K2	4	2 clusters, 2.6	0.212
B3	3	1.7, NA	0.214
B6	2	20.2	0.226
P2	2	cluster	0.302
B8	4	cluster of 3, 7.9	0.376
K4	5	cluster, 2.0–6.4	0.396
B4	2	22.1	0.406
K1	2	6.6	0.428

TABLE 4. The results of a Mantel test of the comparison of the genetic and spatial distance matrices.

Site name	Product-moment correlation	Interpretation	Probability (<i>p</i>)
Paulina View	0.13715	very poor fit	0.257
Broken Top	0.07162	very poor fit	0.458
Katati-2	0.19683	very poor fit	0.447

ten cases. Even though species of *Botrychium* are considered mainly self-fertilizing, we chose to test the probability of shared genotypes being the result of random mating. This would give a conservative value of the potential establishment of disjunct shared genotypes by spores, which would only be higher with increased inbreeding. The probability of these ten genotypes occurring a second time in each population by random mating (Parks and Werth, 1993) was relatively high, ranging from 0.097945 to 0.427633 (Table 3). Two shared genotypes were represented by a pair of individuals each belonging to a different population. Because of the large geographic distance between these plants, they were not considered potential clones resulting for the dispersal of gemmae.

The Mantel test was used to test for correlations between the matrix of genetic diversity and spatial distance. All three populations have a very poor fit (Table 4). Genetic diversity did not correlate with spatial distance in *B. pumicola*.

Because isozyme studies of other species of *Botrychium* have demonstrated high degrees of inbreeding (McCauley, Whittier, and Reilly, 1985; Soltis and Soltis, 1986; Hauk and Haufler, 1999), the genetic diversity statistics (G_{st}) were calculated twice. Once with the assumption of Hardy-Weinberg equilibrium ($F_{is} = 0$) and once with the assumption that the populations are mostly selfing ($F_{is} = 0.95$). The among population differentiation estimates under both of these assumptions was low, $G_{st} = 0.1147$ (Hardy-Weinberg equilibrium) and $G_{st} = 0.0950$ ($F_{is} = 0.95$). These low values indicate that most of the genetic diversity of *B. pumicola* is found within populations and there is little among-population differentiation. Similarly, an UPGMA analysis did not segregate individuals by the three populations (not shown).

Population-level genetic diversity statistics are summarized in Table 5. The least genetically diverse population is Katati with 34% polymorphic loci. Both Nei's genetic diversity and Shannon diversity were calculated with $F_{is} = 0.95$. These indexes are influenced by the deviation from Hardy-Weinberg equilibrium. However, the same trends in diversity are seen if Hardy-Weinberg equilibrium is assumed. In general the ISSR genetic diversity within a site of *Botrychium pumicola* is high when compared with isozyme results of other species of *Botrychium* (McCauley, Whittier, and Reilly, 1985; Soltis and Soltis, 1986; Soltis, Soltis, and Holsinger, 1988; Watano and Sahashi, 1992; Hauk and Haufler, 1999).

TABLE 5. Measures of genetic diversity in each population of *Botrychium pumicola*. $F_{is} = 0.95$.

Site name	Proportion of distinguishable genotypes	Polymorphic loci	<i>h</i>	<i>I</i>
Paulina View	0.93	45%	0.1445	0.2196
Broken Top	0.90	48%	0.1622	0.2455
Katati-2	0.65	34%	0.1027	0.1588

Note: *h* = gene diversity, *I* = Shannon's index.

DISCUSSION

Inter-simple sequence repeats are presumably noncoding loci and therefore have less mutational constraints than isozyme loci (Wolfe and Liston, 1998). Thus it is not surprising that ISSRs have provided more polymorphisms than isozymes (Fang et al., 1997; Wolfe, Xiang, and Kephart, 1998; Esselman et al., 1999; Ge and Sun, 1999). When compared with isozyme studies of other species of *Botrychium* including *B. pumicola*, ISSR markers show a high degree of genetic diversity in *Botrychium pumicola*. Among the 99 individuals are 71 ISSR types, in contrast no polymorphic isozyme loci have currently been found in *B. pumicola* (Hauk and Haufler, 1999; D. R. Farrar, personal communication, Iowa State University). In fact, no or low levels of isozyme variations appear to be the normal state of a subgenus *Botrychium* population (Farrar, 1998; Hauk and Haufler, 1999). Members of the subgenus *Sceptridium* also exhibit low levels of isozyme variation. Four populations with 184 individuals of *B. virginianum* (L.) Swartz (subgenus *Osmundopteris*) had only four polymorphic loci among 18 loci analyzed (Soltis and Soltis, 1986). McCauley, Whittier, and Reilly (1985) found only five polymorphic loci among nine enzymes from 209 individuals in three populations of *B. dissectum* Sprengel (subgenus *Sceptridium*). Although these authors did not give the total number of genotypes in their examination of these species, there could not be more than 32 and 15 genotypes respectively. Watano and Sahashi (1992) examined four species of *Botrychium* subgenus *Sceptridium*, *B. multifidum* (S. G. Gmelin) Ruprecht var. *robustum* (Milde) Tagawa, *B. nipponicum* (Makino) Holub, *B. triangularifolium* Sahashi, and *B. ternatum* (Thunb.) Lyon. They found *B. ternatum* to be the most polymorphic of the four species. Three populations with 138 individuals included 30 genotypes, only two of which were restricted to a single plant.

The mechanism for maintaining this high level of diversity in *Botrychium pumicola*, a supposedly self-fertilizing plant, requires examination. Before addressing the source of variation, we need to ask whether our data can be used to test the assumptions that *B. pumicola* is indeed self-fertilizing. Although ISSR loci can potentially distinguish many individuals, they unfortunately do not measure true heterozygosity due to their generally dominant inheritance (Wolfe and Liston, 1998). For this reason, the level of inbreeding cannot be determined with ISSR data alone. Based on the results of other studies, inbreeding is expected. Except for one population of *B. multifidum* var. *robustum* (Watano and Sahashi, 1992), all species of *Botrychium* studied with codominant isozyme markers, including *B. pumicola* (Hauk and Haufler, 1999; D. R. Farrar, personal communication), have exhibited high levels of homozygosity (McCauley, Whittier, and Reilly, 1985; Soltis and Soltis, 1986; Hauk and Haufler, 1999). This appears to result from the most extreme type of inbreeding in plants, intragametophytic selfing (Klekowski, 1979). In this type of self-fertilization the gametophyte, even if the parent sporophyte was heterozygous, will develop a homozygous sporophyte. Although it might be expected that this would lead to a population of low genetic diversity, in seed plants it is not uncommon for selfing plants to have high genetic diversity (Hedrick, 1998). There are mechanisms for maintaining polymorphisms such as variable selection over space, which may maintain levels of polymorphisms identical to random mating (Hedrick, 1998). However, no data exist to test this hypothesis in *B. pumicola*.

Alternatively, some ISSR bands may not behave in a Men-

delian manner. For example Tsumura, Ohba, and Strauss (1996) found 3 of the 77 bands studied departed from Mendelian expectations. This suggests a high mutation rate from generation to generation. It is therefore possible that high mutation rates of ISSR loci in *Botrychium pumicola* may account for the genetic diversity observed. The simple sequence repeats, which are the basis for the primer site of ISSRs, are known to have a high rate of gaining and losing repeat units due to DNA slippage (Schlötterer, 1998). Chromosomal structural rearrangements have also been suggested as a source of ISSR variation (Wolfe and Liston, 1998). *Botrychium pumicola* is a diploid with 90 chromosomes (Wagner and Wagner, 1993). One could imagine that these ISSR *B. pumicola* loci may be in portions of the chromosomes that do not segregate independently during meiosis, even in a completely homozygous individual. Further studies are needed to test these possibilities.

It is fairly common for up to seven plants of *Botrychium pumicola* to arise in a cluster from the same point of soil. Plant clusters were expected to have shared genotypes, either because of asexual reproduction of gemmae or through multiple self-fertilization of a single gametophyte. Except through the direct observation of the developing plants, it is impossible to distinguish between the two modes of reproduction, gemmae and multiple self-fertilization. Most of the plant clusters sampled, nine of 13, were composed of plants with identical ISSR type. However, 30% of sampled clusters contain plants of more than one ISSR type, apparently representing independent fertilization events. The fertilizations may be temporally independent or simultaneous. In the latter case, clusters may result from cross-fertilization of adjacent gametophytes. The spores of *B. pumicola* unlike other species of this genus, often remain in a tetrad (W. H. Wagner, personal communication, University of Michigan). This may increase the probability of multiple gametophytes developing in a cluster.

The dispersal of gemmae is important for determining their significance to the population. To determine dispersal, it is better to examine the shared genotypes that are not in a cluster. Ten genotypes fit these criteria (Figs. 1, 2, and 3). None of these were rare in their population and the probability of a second, sexually developed plant was at least 9.8% or greater (Table 3). It is assumed that spatially disjunct plants with identical genotypes, and a 5% or greater chance of being developed from a second sexual event, are not clones (Parks and Werth, 1993; Montalvo et al., 1997). This method assumes that these individuals resulted from random mating, which is not supported by isozyme data. The probability of two different individuals having the same genotype will increase with higher rates of inbreeding. Hence the likelihood of a parent producing an identical offspring should increase the probability of identical genotypes from independent sexual events. Therefore, the probability of shared genotypes arising from spores (Table 3) is a conservative estimate and the true values would be higher.

Most asexual plants that reproduce by stolons or rhizomes are expected to have a patchy distribution of clones. However, plants that produce vegetative diaspores may have more intermingling of clones (Gabrielsen and Brochmann, 1998). The gemmae of *Botrychium pumicola* probably disperse only through soil movement. Animals may be responsible for long-distance dispersal events (e.g., more than a meter). We do not expect these dispersal events to occur often. Instead, we expect the gemmae to disperse only a short distance from the parent plant, producing a patchy distribution of clones. The plant

clusters are an extreme example of this type of distribution. However, none of the disjunct plants sampled less than a meter apart had identical genotypes (Figs. 1, 2, and 3). The Mantel test supported this by showing no correlation between genetic distance and spatial distribution. The data are consistent with the dispersal of gemmae not being an important factor in the population structure of this species.

It is possible that some of these disjunct identical genotypes (Table 3) were formed by self-fertilization of a gametophyte. Intragametophytic self-fertilization can mimic asexual reproduction. It should be easier for a spore to disperse long distances and develop into an identical genotype through self-fertilization than for a gemma to be transported with soil. The self-fertilization of gametophytes could produce the observed pattern of intermingled shared genotypes.

As in the isozyme studies of species of *Botrychium*, *B. pumicola* has a low G_{st} value (McCauley, Whittier, and Reilly, 1985; Soltis and Soltis, 1986; Watano and Sahashi, 1992). This low value showed little genetic differentiation among populations of *B. pumicola*. The lack of interpopulational genetic differentiation in species of *Botrychium* was assumed to be the product of high rates of gene flow due to the long-distance dispersal of spores (Soltis, Soltis, and Holsinger, 1988).

The genetic diversity of rare plant populations concerns natural resource managers. We sampled only three populations of this species, but the Katati-2 population had a lower genetic diversity than the other two. Several reasons may account for this, including sampling size. However, this population does stand out in two ways that need further investigation. The Katati-2 population is in a montane habitat. The subalpine sites typically have more plants than the montane sites (Joslin, 1997). This trend is observed in the three populations of this study (Table 1). The Katati-2 population may be less diverse because of fewer individuals contributing to the gene pool. The other difference in the Katati-2 population is the recent disturbance of salvage wood cutting (Joslin, 1997). Species of *Botrychium* are known to favor disturbed sites (Wagner and Wagner, 1993). The spores need prolonged periods of darkness to germinate (Whittier, 1973), which may be facilitated by soil disturbance. Why this might decrease genetic diversity is unclear; perhaps it results from a more recent colonization of the site.

The main goal of this research was to evaluate the significance of asexual reproduction of gemmae of *Botrychium pumicola*. ISSR bands have provided useful genetic markers for examining the population structure of this species. There were few shared genotypes within the populations. Over half of the shared genotypes were spatially disjunct. Because of the high probability of a second sexual occurrence of these genotypes, especially when self-fertilization occurs, and the lack of a patchy pattern of genotypes expected from the distribution of gemmae, we assume that these shared genotypes are independent sexual events and not the result of reproduction by gemmae. Gemmae are probably important in the temporal maintenance of a genet. The half-life of species in the subgenus *Botrychium* is short, ranging from 1.3 yr (Muller, 1993) to ~3 yr (Lesica and Ahlenslager, 1996). It needs to be determined how well gemmae can perpetuate a genet.

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