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RAPD VARIATION AMONG AND WITHIN SMALL AND LARGE POPULATIONS OF THE RARE CLONAL PLANT *RANUNCULUS REPTANS* (RANUNCULACEAE)[1]

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In the pre-alpine region of Europe numbers and sizes of populations of the clonal lake shore plant *Ranunculus reptans* have declined because of the regulation of lake water levels. We investigated genetic variation among and within 17 populations of different size (cover 1–10 000 m^2) in *R. reptans* with RAPD (random amplified polymorphic DNA) profiles. We sampled 127 rosettes in 14 populations at Lake Constance and three populations at or near Lake Como. There was significant genetic variation between plants from the two lake regions (5.9%, analysis of molecular variance [AMOVA], $P < 0.001$), among populations within lake regions (20.4%, $P < 0.001$), and within populations (73.7%, $P < 0.001$). Under the assumptions of Wright's island model the variation among populations corresponds to a gene flow of $N_em = 0.70$. Within the 14 Lake Constance populations we detected significant genetic variation among subpopulations separated by only a few metres (4.0% of the within-population variation; $P < 0.05$). Molecular variance was 24% smaller in small populations covering <100 m^2 area than in larger ones ($P < 0.03$), indicating that samples from large populations were genetically more variable than samples representing comparable areas of smaller populations. We conclude that gene flow among populations is very limited and that genetic drift has caused reduced genetic variability of smaller populations. Conservation of genetic variability in *R. reptans* requires persistence of large and also of small populations (because of population differentiation), and it could be enhanced by increasing the size of small populations (to counter genetic drift).

Key words: conservation genetics; gene flow; genetic drift; molecular variation; population-genetic structure; population size; Ranunculaceae; *Ranunculus reptans*; RAPD-PCR; rare plant.

Regulation of lake water levels has decreased the numbers and sizes of populations of the lakeshore plant *Ranunculus reptans* in Central Europe, where it is now considered endangered (Landolt, 1991; Korneck, Schnittler, and Vollmer, 1996). We investigated both differentiation among populations and variability within populations of this rare outcrossing clonal plant at the molecular level. The amount and partitioning of genetic variation among and within populations result from the dynamic processes of gene flow, selection, inbreeding, genetic drift, and mutation (Hartl and Clark, 1994). Thus, knowledge of the current genetic structure allows inferences about past processes. At the same time genetic variation represents the starting point for further evolution and is an important prerequisite for the prediction of evolutionary responses. This is of practical significance in the light of human-caused habitat fragmentation, alteration, or destruction.

Levels of gene flow among populations of many plant species are low (Levin, 1984; Slatkin, 1985). This allows differentiation among populations. Such differentiation may be related to geographic distance among populations if the spatial pattern reflects the colonization history, if a formerly larger population was fragmented or if current gene flow makes populations closer to each other more similar than more distant ones. Limited gene flow on the smaller within-population scale can lead to small neighborhood sizes and thus to populations that consist of genetically differentiated subpopulations (Wright, 1951; Slatkin, 1985).

Genetic structure among and within populations also depends on the life history of a species. Outcrossing species tend to have higher levels of variability within populations but smaller degrees of differentiation among populations than selfing species (Hamrick and Godt, 1990; Schoen and Brown, 1991). In annuals a higher degree of differentiation is found compared with perennials. Species reproducing only sexually show larger differentiation than species that reproduce both sexually and asexually (Hamrick and Godt, 1990). From these patterns it may be expected that outcrossing clonal plants show little differentiation among populations. Studies of genetic variability of clonal plants show that their populations usually consist of several to many genetic individuals and levels of within-population genetic variability in clonal plants are similar to those in nonclonal plants (Ellstrand and Roose, 1987; Widén, Cronberg, and Widén, 1994; McLellan et al., 1997).

In the last decades changes in land use have caused the alteration and fragmentation of many habitats. Therefore, nowadays populations of many plant species are smaller and more isolated from each other than in the past. Decreased size and increased isolation of remnant populations after such changes may have important consequences for the genetic structure of plants. Gene flow may be decreased because of increased geographic isolation. Accordingly, pronounced genetic differentiation among populations has been reported for a number of rare species (e.g., Brauner, Crawford, and Stuessy, 1992; Raijmann et al., 1994; Travis, Maschinski, and Keim, 1996; Fischer and Matthies, 1998a). In isolated populations genetic drift may eventually reduce genetic variation (Lacy, 1987; Ellstrand and Elam, 1993; Frankham, 1996), especially because effective population sizes are usually much smaller than the number of reproductive individuals in a population (Crawford, 1982; Frankham, 1995). Because genetic drift diminishes genetic variation in a manner inversely proportional to effec-

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tive population size in each generation (Hartl and Clark, 1994) drift is most likely to be observed in small populations of short-lived plants (Hamrick et al., 1991). From these considerations it may be hypothesized that effects of population isolation and genetic drift are not very pronounced in outcrossing clonal plants.

Per definition genetic drift is selectively neutral. Therefore it is best measured using selectively neutral genetic markers such as RAPD (random amplified polymorphic DNA; Williams et al., 1990). Once established, RAPD-PCR (polymerase chain reaction) has the advantage of being quick and easy, requiring little plant material, and having a high resolution (e.g., Steinger, Körner, and Schmid, 1996; Gugerli, Eichenberger, and Schneller, 1999). Thus, it is especially appropriate for studies of rare plants and has successfully been used to demonstrate a positive relationship between population size and genetic variability in a rare plant, indicating genetic drift (Fischer and Matthies, 1998a).

Changes in genetic variability because of habitat fragmentation are relevant for plant conservation because they may affect plant fitness (Young, Boyle, and Brown, 1996; Fischer and Matthies, 1998b). Though genetic drift is selectively neutral, it may negatively feed back on plant fitness via indirect pathways such as increased inbreeding in combination with inbreeding depression (Ellstrand and Elam, 1993), increased susceptibility to pathogens (Schmid, 1994), accumulation of deleterious mutations (Lande, 1995), and because of the reduced potential to adapt to changing environmental conditions.

We studied the self-incompatible stoloniferous buttercup *Ranunculus reptans.* Its populations close to the Alps most likely represent relicts of a formerly more widespread distribution in this area (Prati and Peintinger, 2000). It occurs on lake shores where most interspecific competition is removed by yearly inundation. From the competition-free zone it scarcely penetrates into closed vegetation farther inland. Regulation of water levels of most lakes has restricted *R. reptans* to small and isolated populations in Central Europe, although it is still common in Northern Europe, >1000 km north of Switzerland (Prati and Peintinger, 2000). Both north and south of the Alps, where it is at the southern limit of its distributional range, most remnant populations are small and are typically separated by several to many kilometres (Prati and Peintinger, 2000). Thus, in the alpine region *R. reptans* is a good example of a species whose geographical range and population sizes have declined because of human interference.

To investigate differentiation among populations and variability within populations of *R. reptans* we studied variation in RAPD profiles of plants from 17 populations of different sizes. Fourteen of these populations were situated at Lake Constance, three at Lake Como. We addressed the following questions: (1) Is there significant genetic variation between populations originating from Lake Constance and Lake Como, among populations from different parts of Lake Constance, among populations within parts, and among subpopulations? (2) How much gene flow occurs among populations and do genetic distances among populations correspond to geographic distances? (3) Is there less genetic variation among plants within small populations than within large populations?

MATERIALS AND METHODS

Study species—*Ranunculus reptans* L. (Creeping Spearwort) is a stoloniferous species of the Ranunculaceae that is closely related to *R. flammula.* The potential distributional range of *R. reptans* comprises the temperate and boreal northern hemisphere (Hess, Landolt, and Hirzel, 1980). In Asia the species is limited to the boreal-arctic zone at latitudes >50° N (Meusel, Jäger, and Weinert, 1965). In North America it ranges from the arctic Canada and Newfoundland south to New Jersey, Pennsylvania, and Michigan, in the Rocky Mountains south to Colorado, and in the west south to California (Britton and Brown, 1913; Flora of North America Editorial Committee, 1997). In Europe its potential range includes Central and Northern Europe with Iceland, Scandinavia, and the Baltic countries as main centres (Meusel, Jäger, and Weinert, 1965; Prati and Peintinger, 2000).

The species, a weak competitor, is restricted to gravel lake shores which are essentially free of reed, woody species, or graminoids (Lang, 1967). At such lake shores the species occurs in temporarily flooded areas. Whereas *R. reptans* is able to survive summer inundation for 3–5 mo, competing grasses and sedges are suppressed by regular flooding. At Lake Constance, *R. reptans* occurs with four species that are considered endemic for the alpine region (Korneck, Schnittler, and Vollmer, 1996): *Deschampsia littoralis, Myosotis rehsteineri, Armeria purpurea,* and *Saxifraga oppositifolia* subsp. *amphibia* (Lang, 1967) of which the latter two are almost extinct (Thomas et al., 1986). These endemic species are seen as glacial relicts (Lang, 1967), i.e., they are assumed to have been widespread during the postglacial period ~10 000 yr ago. Although there is no direct evidence that *R. reptans* also is a relict species, its disjunct nordic-alpine distribution (Jalas and Suominen, 1988) may be interpreted as remnant from a formerly closed distributional range, which had been diminished with postglacial reforestation.

Ranunculus reptans reproduces vegetatively by secondarily rooting nodes along horizontally growing stems. Vegetative reproduction may lead to high ramet densities of *R. reptans* in the field. The number of ramets in populations of *R. reptans* can fluctuate strongly between years. On average, plants of the self-incompatible *R. reptans* produce one insect-pollinated flower on every third node (Prati and Peintinger, 2000). Average infructescences contain 10–20 seeds. While seedling recruitment has been observed within established populations (Prati and Peintinger, 2000) its importance relative to vegetative recruitment is unclear. Stolons of *R. reptans* with several rosettes may become detached by the breaking force of the waves and have been found floating (Schröter and Kirchner, 1902, p. 44; M. Peintinger, personal observation). Thus, *R. reptans* potentially appears to be able to float large distances.

Study area and plant material—In spring 1995 and 1997 we sampled plants from 14 populations of *R. reptans* north of the Alps at Lake Constance in Germany, Austria, and Switzerland. The Rhine River enters Lake Constance close to its eastern end. It reaches the part called Untersee after passing a 6-km-long geographic bottleneck (Fig. 1) and leaves the lake at its western end. Six of our sampling populations are situated at Untersee. The other eight populations are situated at the main part of the lake, which consists of Obersee and Überlingersee (Fig. 1).

At Lake Constance the *R. reptans* habitats are ~10-m-wide stripes of gravel shore parallel to the winter water line. In each Lake Constance population we selected one 20-m-long stretch within the area colonized by *R. reptans.* In ten populations we sampled 4–5 rosettes along these stretches in distances of 5 m of each other. Then we collected another 4–5 plants 5 m farther inland. In this way we collected 8–10 rosettes per population representing a 5 × 20 m area. In four populations we sampled 8–10 rosettes at interdistances <5 m because their total area was <5 × 20 m. In all Lake Constance populations our sampling allowed us to distinguish subpopulations by comparing the left half of a population with the right half (left and right as seen from the lake).

In June 1997 we sampled plants from another three populations south of the Alps at Lake Mezzola and Lake Como in Italy, ~175 km south of Lake Constance (Fig. 1). Two of these populations are situated close to the village Dascio in the very south of Lake Mezzola at the east and west shores of the Mera River where it leaves the lake. One of these populations (Dascio 1) colonized a small sand shore of 10 m length where we sampled plants at interdistances of 2–3 m. The other colonized a sandy bay of ~30 × 30 m surrounded by reed that showed no zonation. There we sampled eight plants at distances of 5 m from each other. The third Italian population (Piano), is situated 3.5 km in the southwest of Dascio between the points where the Mera

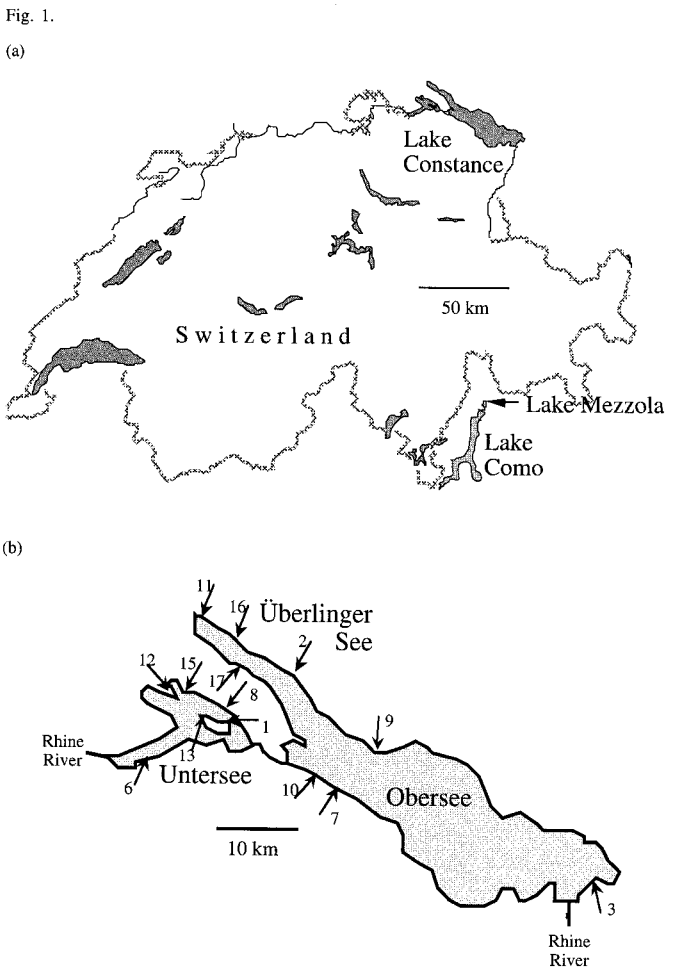

Fig. 1. Map of (a) Switzerland and adjacent area with Lake Constance, Lake Como, and Lake Mezzola, and (b) the study populations of *Ranunculus reptans* at Lake Constance.

River and Adda River are flowing into Lake Como. It colonized a wet meadow of high vegetation coverage, which is separated from the lake only by a small bank. There we sampled eight plants at distances of 5 m from each other. We kept collected plants in a plant room under artificial light.

To obtain a measure of population size we estimated the area of every patch occupied by *R. reptans* and its percentage of ground cover in each Lake Constance population in November 1997. Then we estimated the area completely covered by *R. reptans* by summing the product of area and ground cover over all patches per population. In the three Italian populations the corresponding area was estimated visually at the time of sampling in June 1997. Population areas ranged over four orders of magnitude between 1 and 10 000 m^2. Because the method and time of estimating population area were different for populations in the two lake regions we classified populations in two size categories and considered eight populations with area-times-cover values <100 m^2 as small and nine >100 m^2 as large. However, levels of significance of correlations between population area and genetic variability of a population (see below) remained the same when we used the numerical estimates of population area.

RAPD-PCR—For the RAPD analysis we used 7–9 plants per population (three for population ''Dascio2'' at Lake Como because five plants died before analysis; Table 1). Vegetative offspring of 32 plants sampled in four populations at Lake Constance in 1995 had been used for several experimental studies of phenotypic and genetic variation in clonal growth of *R. reptans* (Prati, 1998). In 1996 these plants had been analyzed with ten RAPD primers (primers 1, 2, 4, 5, 6, 7, 11, 13, 17, 18 of kit B, Operon Technologies, Alameda, California, USA) for a comparison of the partitioning of molecular genetic variation and of quantitative variation (Prati, 1998). In September 1997 we cut leaves from 95 plants of the 13 other populations, and as control for the repeatability of the RAPD procedure from four plants of the four populations analyzed earlier. Leaves were rinsed with distilled water, put into Eppendorf tubes, lyophilized, and stored at -18°C. DNA was extracted using a modified Rogers and Bendich (1988) procedure (for a detailed description see Steinger, Körner, and Schmid, 1996). We resuspended extracted DNA in Tris-EDTA buffer (1 mmol/L Tris-HCl, pH 8.00 and 0.1 mmol;/L EDTA, pH 8.00), determined the DNA concentration for each sample with a fluorometer (Hoefer TKO 100, Hoefer Scientific Instruments, San Francisco, California, USA), and standardized it to 4×10^{-9} g DNA per 10^{-6} L buffer. In October and November 1997 RAPD-PCR was performed exactly as outlined in Steinger, Körner, and Schmid (1996) with the same ten primers as for the 32 plants analyzed earlier (Prati, 1998).

We visually scored the presence or absence of bands for all 127 plants using printed images of the electrophoresis gels. For the 32 plants analyzed earlier we used the images of Prati (1998). RAPD patterns of the four control plants were consistent with the prior analysis. However, some of the bands scored by Prati (1998) could not be scored for all of the 95 new plants because the range of intermediate molecular weight with good resolution (Stewart and Porter, 1995) was not exactly the same as in the first analysis. On the other hand a few bands that were monomorphic among the four populations turned out to be polymorphic when all plants were considered. The final presence–absence matrix contained scores at 38 polymorphic band positions for each of the 127 individuals (primer 1—3 bands; 2—2 bands; 4—6 bands; 5—4 bands; 6—2 bands; 7—6 bands; 11—4 bands; 13—2 bands; 17—5 bands; 18—4 bands).

Statistical analysis—To illustrate relatedness among individuals and among populations we analyzed the presence–absence matrix of RAPD bands with Ward's hierarchical clustering (implemented in JMP 3.1, SAS Institute, Cary, North Carolina, USA). Variation in RAPD patterns was analyzed by analysis of molecular variance (program AMOVA, version 1.55; Excoffier, Smouse, and Quattro, 1992; Stewart and Excoffier, 1996). With AMOVA we calculated variance components and their significance levels for variation between plants from the two lakes, among three distinct regions of Lake Constance, among populations within regions, and within populations using RAPD data of all 127 plants. Pairwise genetic distances (ϕ_{st}) among the 17 populations and their levels of significance were also obtained from AMOVA. Under the assumptions of Wright's island model, gene flow (number of migrants per generation $= N_e m$) can be approximated from AMOVA Φ statistics (analogous to F statistics) as $N_e m = (1/4)\,[(1/\phi_{st}) - 1]$, with effective population size N_e and migration rate m (Wright, 1951; but see Whitlock and McCauley, 1999). A Mantel test was used to test whether matrices of genetic distances between populations were significantly correlated with matrices of geographic distances (1000 permutations; routine MXCOMP of the NTSYS-pc package; Rohlf, 1994).

To estimate genetic variability within populations we only used plants sampled at 5-m distances from each other, i.e., we omitted 20 plants from the four small populations where some plants were sampled <5 m from each other. Thus, the reduced sample (107 of 127 plants) for the analysis of population variability represented similar sampling densities in all populations. As a measure of genetic variability per population we calculated molecular variance for each population as AMOVA sum of squares divided by $n - 1$ (Fischer and Matthies, 1998a). Molecular variance was independent of the number of analyzed plants per population ($N = 17$, $r^2 = 0.033$, $P > 0.48$). Homogeneity of molecular variance in pairs of populations was tested with Bartlett tests implemented in the AMOVA 1.55 program.

RESULTS

Genetic distances and variance partitioning—The 127 plants of *R. reptans* belonged to 124 different RAPD phenotypes. Only in the two small Lake Constance populations,

TABLE 1. Study populations of *Ranunculus reptans.* Population numbers are given after the location in parentheses. The number of sampled plants indicates how many plants were used in the analysis of differences among populations. Between parentheses we give the number of plants used in the analysis of variability within populations. L denotes populations that completely covered >100 m² in fall 1997, and S denotes populations that covered <100 m².

Site	Latitude (north)	Longitude (east)	No. of sampled plants	Molecular variance	Population size
Bodensee, Obersee					
Landschlacht (10)	47°37′36″	9°15′27″	7 (5)	3.50	S
Bregenz (3)	47°30′21″	9°42′59″	8	4.61	L
Güttingen (7)	47°37′02″	9°17′06″	7	3.81	L
Immenstaad (9)	47°39′48″	9°20′49″	8 (4)	4.00	S
Bodensee, Überlingersee					
Birnau (2)	47°45′00″	9°12′38″	7	6.48	L
Ludwigshafen (11)	47°49′09″	9°02′75″	8 (4)	4.33	S
Sipplingen (16)	47°47′32″	9°06′37″	8 (3)	2.67	S
Wallhausen (17)	47°44′58″	9°08′24″	8	4.71	L
Bodensee, Untersee					
Reichenau-Bibershof (1)	47°41′24″	9°05′42″	8	3.70	L
Radolfzell-Mettnau (12)	47°43′16″	9°00′39″	7	4.90	S
Reichenau-Mutschellern (13)	47°42′13″	9°02′48″	7	2.90	S
Allensbach-Schlafbach (15)	47°33′40″	9°01′36″	8	3.75	L
Glarisegg (6)	47°39′27″	8°57′20″	9 (5)	2.10	S
Hegne (8)	47°42′16″	9°05′57″	8	4.21	L
Lake Como region					
Piano (14)	46°09′54″	9°23′20″	8	4.36	L
Dascio1 (4)	46°11′33″	9°25′36″	8	3.13	S
Dascio2 (5)	46°11′32″	9°25′36″	3	5.00	L

which consisted of three (Sipplingen) and four (Immenstaad) distinct patches of *R. reptans*, three pairs of plants (two in population ''Immenstaad,'' one in ''Sipplingen'') showed identical RAPD phenotypes. In these cases pairs had been sampled within 25 cm from each other in the same patch of *R. reptans*, suggesting that sampled pairs belonged to the same genet.

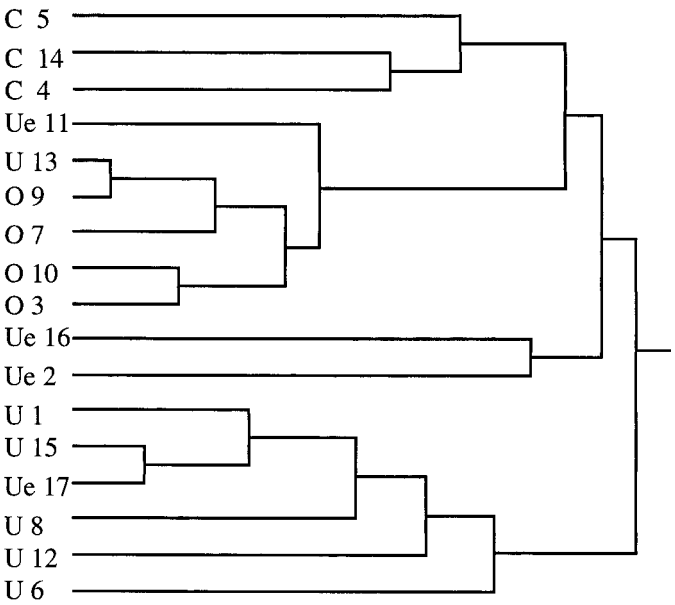

Fig. 2. Ward dendrogram of RAPD variation among 127 plants of 17 populations of *Ranunculus reptans.* Populations are numbered as in Table 1. C denotes populations at Lake Como and Lake Mezzola, O at Obersee, U at Untersee, and Ue at Überlingersee (see Fig. 1). Clustering is based on mean RAPD phenotypes per population.

However, all plants sampled in the other patches in these two small populations showed different RAPD phenotypes, suggesting that different clones occur even in small patches in the smallest populations. The maximum genetic distance between two plants was 25 bands (i.e., plants differed in 25 of the 38 scored band positions). This was the distance between a plant from ''Birnau'' at Lake Constance and a plant from ''Piano'' at Lake Como. The largest distance between two plants from Lake Constance was 24 bands between a plant from ''Birnau'' at the northern shore and one from ''Güttingen'' at the southern shore.

Of the 136 pairwise genetic distances (pairwise ϕ_{st}) between pairs of the 17 populations, 99 were significant, i.e., more than expected for a random distribution. Moreover, 83 distances were significant at the 0.1% level (Appendix 1).

The three populations of *R. reptans* at Lake Como formed a distinct cluster in the cluster analysis of mean RAPD phenotypes per population (Fig. 2). Correspondingly, there was significant RAPD variation between Lake Constance populations and Lake Como populations (analysis of molecular variance, 5.89%; $P < 0.05$, Table 2A).

Differentiation among the three regions of Lake Constance (Untersee, Obersee, and Überlingersee) was reflected in highly significant among-region RAPD variation of Lake Constance plants (8.73%, $P < 0.001$, Table 2B). The six populations at Untersee (Fig. 1b) were significantly different from the eight populations at the main part of Lake Constance (Untersee vs. rest of the lake explained 9.24% of the RAPD variation of plants sampled in these two regions; AMOVA, $P < 0.001$). This effect summarizes that the six populations at Untersee differed from the four populations at Obersee (Untersee vs. Obersee, 11.8%, $P < 0.001$) and from the four at Überlingersee (Untersee vs. Überlingersee, 8.35%, $P < 0.012$). Within

TABLE 2. Summary of nested analysis of molecular variance (AMOVA). Plants represented 17 populations of *Ranunculus reptans*. The analysis is based on RAPD phenotypes consisting of 38 band states. Levels of significance are based on 1000 iteration steps. Fixation indices: Φ_{st}, correlation among random RAPD phenotypes within populations relative to the correlation of random pairs drawn from the whole sample; Φ_{ct}, correlation among random RAPD phenotypes within regions relative to the correlation of random pairs drawn from the whole sample; Φ_{sc}, correlation among random RAPD phenotypes within populations relative to the correlation of random pairs drawn from the region. (A) Two regions: Lake Constance vs. Lake Como, (B) Three regions within Lake Constance (Obersee, Untersee, and Überlingersee).

Level of variation	df	Variance component		Fixation index	*P*
		Absolute	%		
A)					
Among regions	1	0.311	5.89	$\Phi_{ct} = 0.059$	<0.042
Among populations	15	1.075	20.38	$\Phi_{sc} = 0.217$	<0.001
Within populations	110	3.888	73.73	$\Phi_{st} = 0.263$	<0.001
Total	126		100		
B)					
Among regions	2	0.453	8.73	$\Phi_{ct} = 0.087$	<0.001
Among populations	11	0.848	16.35	$\Phi_{sc} = 0.179$	<0.001
Within populations	94	3.887	74.91	$\Phi_{st} = 0.251$	<0.001
Total	107		100		

the main part of the lake differences between the regions Obersee and Überlingersee were marginally significant (eight populations, 4.84%, $P < 0.083$).

We found 20.4% of RAPD variation among all 17 populations within regions ($P < 0.001$, Table 2A), and 73.7% within populations ($P < 0.001$). Within the 14 Lake Constance populations there was significant RAPD variation between subpopulations (i.e., left half vs. right half of sampling areas, AMOVA, 4.0% of 74.9% within-population differentiation, $P < 0.031$), indicating genetic differentiation even within populations at a small spatial scale of only a few metres.

Correlation of geographical and genetic (RAPD) distances—Straight geographic distance did not explain genetic distance among populations: neither the matrix of 136 pairwise genetic distances (ϕ_{st}) among all 17 populations ($r = -0.0687$, Fig. 3) nor of 91 distances among the 14 populations at Lake Constance ($r = -0.0011$) was significantly correlated with corresponding matrices of geographic distances (Mantel tests). Thus, observed differentiation among regions and among populations (Table 2, Appendix 1) did not directly correspond to geographic distance.

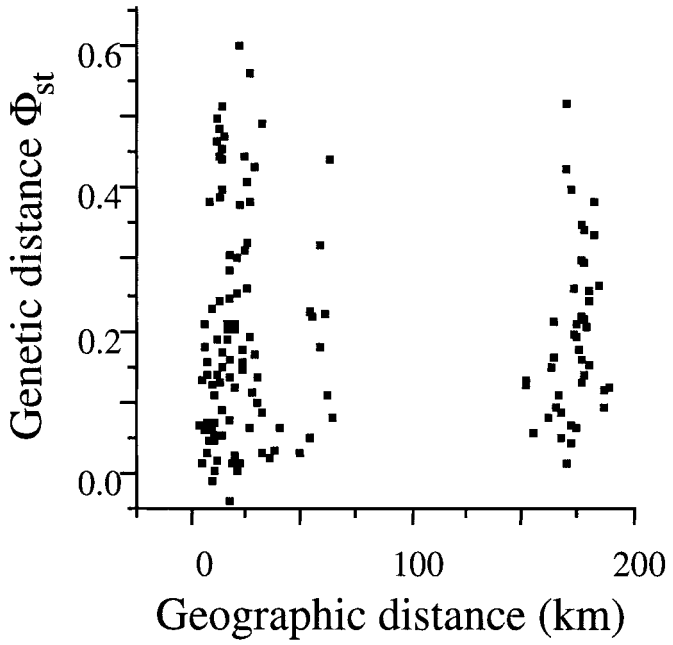

Fig. 3. The relationship between pairwise geographic and pairwise genetic distances (Φ_{st}) among 17 populations of *Ranunculus reptans*. Distances >100 km denote pairs with one population at Lake Constance and the other at or near Lake Como.

Genetic distance among the 14 Lake Constance populations was also independent of two further measures of geographic distance (distance along the lake shore, $r = 0.062$; east–west distance corresponding to the flow direction of river Rhine through Lake Constance, $r = -0.031$).

Molecular variation and population size—Molecular variance within populations was significantly different among the 17 populations ($P < 0.001$; Bartlett test). Of the 136 pairwise Bartlett tests of homogeneity of population variation, 103 were significant, 84 of them at the 0.1% level (Appendix 2). There was no difference between mean molecular variance of Lake Constance populations ($N = 14$; mean = 3.98) and of Lake Como populations ($N = 3$; mean = 4.16)

Mean molecular variance of Lake-Constance subpopulations did not differ from the whole population value (14 populations, paired *t* test, $P > 0.124$), suggesting that molecular variance is a good per-area measure of genetic variability. Molecular variance was 24% smaller in small populations covering <100 m² area than in larger ones ($N = 17$; $P < 0.05$; Fig. 4), i.e., samples from large populations were genetically more variable than samples representing comparable areas of smaller populations. At Lake Constance molecular variance of the seven smaller populations covering <100 m² area was marginally significantly smaller (−21.9%) than that of the seven large populations ($P < 0.09$).

DISCUSSION

Analysis of genetic variation—The variance of population genetic estimates does not decrease substantially if more than 30 RAPDmarkers are used (Aagard, Krutovskii, and Strauss, 1998). Therefore we think that the number of 38 markers used in our study is appropriate. With these 38 polymorphic markers obtained with ten primers we could differentiate 124 RAPD phenotypes among 127 studied plants. All three pairs

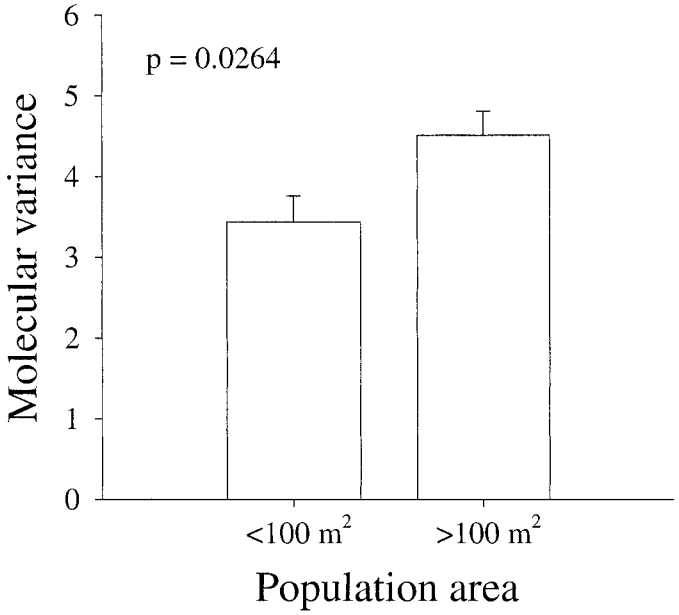

Fig. 4. The relationship between population size (area completely covered by population in fall 1997) and molecular variance in *Ranunculus reptans.* Sampled plants from large populations represented comparable areas as samples of smaller populations.

of identical RAPD phenotypes were found in the two smallest populations in small patches densely crowded with *R. reptans* within 25 cm distance from each other. In glasshouse experiments clones of *R. reptans* grew farther than 25 cm in several weeks (Prati, 1998). We consider it highly likely that the three pairs of identical phenotypes represented three clones. Therefore we are confident that the resolution in our study was sufficient to distinguish all genotypes. It was not our aim to assess clone size in natural populations of *R. reptans*. However, apparently, clones of this species may extend over at least 25 cm. Nevertheless, in similar patches in the same populations sampled pairs belonged to different genotypes, suggesting that different clones occur even in small patches in the smallest populations. Very high densities of genotypes have also been found for *Trifolium repens,* which like *R. reptans* has a clonal guerilla growth strategy (Cahn and Harper, 1976; Harper, 1983), while the phalanx species *Carex curvula* showed a very low density of genotypes (Steinger, Körner, and Schmid, 1996).

The analysis of molecular variance indicated pronounced genetic differentiation among populations of *R. reptans.* There has been some debate over the estimation of population genetic parameters from RAPD data because the inference of F_{st} from the generally dominant RAPD data requires two assumptions, namely that null bands are homologous and that populations are in Hardy-Weinberg equilibrium (Lynch and Milligan, 1994; Ayres and Ryan, 1999). However, the high levels of significance in our study suggest that our results are rather robust to deviations from these assumptions. Moreover, self-incompatibility of *R. reptans* may suggest that populations do not strongly deviate from Hardy-Weinberg equilibrium. Concerns were also raised that RAPD-based estimates of differentiation may be biased towards higher values compared with estimates based on other markers, and it was suggested using 100 plants per population and restricting data sets according to the ''3/N-criterion,'' i.e., to exclude markers with very low frequencies of null alleles (Lynch and Milligan, 1994; see Isabel et al., 1999, for an application). However, genetic distances between 11 populations of *Hippophae rhamnoides* represented by about ten plants each were consistent between the complete and the restricted data set (Bartish, Jeppson, and Nybom, 1999). Moreover, these authors conclude that in small samples of closely related populations, the 3/N-criterion may even underestimate mean genetic diversity among populations, while it may be appropriate in analyses with larger population samples.

Genetic differentiation among populations and subpopulations—Similarly to our study, pronounced genetic differentiation among populations was found in several rare plant species and attributed to low or absent interpopulation gene flow (Schmid, 1984, 1986; Brauner, Crawford, and Stuessy, 1992; Dolan, 1994; Raijmann et al., 1994; Travis, Maschinski, and Keim, 1996; Fischer and Matthies, 1998a). Observed genetic differentiation among populations of *R. reptans* both between and within regions (Table 2) suggesting low gene flow among populations is in accordance with the geographic isolation of the populations. Generally, distances between populations of *R. reptans* are large, and the median distance to the closest population in our sample was 3.1 km (4.0 km if only Lake Constance populations were considered). Frequently the area between lakes and between populations along lake shores appears not suitable for pollinator movement. This situation is typical for lakeshore plants, whose linear habitats represent small islands frequently disrupted by reed belts or settlements (Lang, 1967).

We found 25–26% of RAPD variation among populations and 74–75% within populations (Table 2). In their review comprising >400 plant species, Hamrick and Godt (1990) used G_{st} values to indicate the proportion of isozyme diversity residing among populations. They report an average G_{st} of 22% for perennial herbs compared with 36% for annuals, an average G_{st} of 23% for sexually reproducing plants compared with 21% for species that reproduce both sexually and asexually, and an average G_{st} of 20% for animal-pollinated outcrossers compared with 51% for selfers. To date, RAPD-based G_{st} values are available for 35 plant species, with an average of 19.3% for 29 outbreeding species, and 62.5% for six inbreeding species (Bussell, 1999). Compared with these values the populations of *R. reptans* are slightly more differentiated than expected for an insect-pollinated clonal outcrosser. In a comparative biosystematic study of five taxa of the clonal *Carex flava* group, phenotypic and genetic variation among populations decreased along the *r-K* continuum and was most pronounced for *Carex viridula* subsp. *viridula,* the most *r*-selected species with the shortest life span and smallest size of clones and tillers, which also had the smallest effective population size (Schmid, 1986; Baur and Schmid, 1996). Habitats, life-history attributes, and pronounced differentiation among populations of *Carex viridula* subsp. *viridula* correspond well with those of *R. reptans.*

We also found significant genetic differentiation among subpopulations within populations on a scale of several metres. This suggests very limited seed dispersal and short dispersal distance of pollen even within populations (Starfinger and Stöcklin, 1996). Moreover, seedling recruitment may be a rather rare event in *R. reptans* (Prati and Peintinger, 1999). Observed small-scale differentiation in RAPD variation within

populations indicates that gene flow would not be sufficient to counteract effects of selection if they would lead to small-scale differentiation also in fitness-related traits (Levin, 1988).

Genetic and geographic distance—We found significant genetic variation at all hierarchical levels, i.e., between Lake Como and Lake Constance, among different regions at Lake Constance, among populations within these regions, among subpopulations within these populations, and within subpopulations. However, the differentiation between populations from Lake Constance and those from the Lake Como region was not very strong and not more pronounced than the differentiation within Lake Constance between Untersee and the main part of the lake. That populations at Untersee were clearly differentiated from the rest of Lake Constance corresponds well with the geographic bottleneck constituted by the 6-km long stretch of the Rhine River between these two parts of the lake (Fig. 1).

Despite differentiation among regions and among populations, genetic differentiation among populations was not related to geographic distance in *R. reptans.* Similarly, no correspondence between geographic and genetic distances has been found in the rare endemic *Rutidosis leptorrhynchoides* (Leeton and Fripp, 1991), in the short-lived monocarpic forbs *Gentianella germanica* (Fischer and Matthies, 1998a) and *Pedicularis palustris* (Schmidt and Jensen, 2000), in the clonal grass *Festuca ovina* and in the perennial forb *Lychnis viscaria* (Berge, Nordal, and Hestmark, 1998). In contrast, significant correlations between genetic and geographic interpopulation distances have been found in the rare perennial *Tradescantia hirsuticaulis* (Godt and Hamrick, 1993), the rare clonal perennials *Helonias bullata* (Godt, Hamrick, and Bratton, 1995) and *Wyetha reticulata* (Ayres and Ryan, 1999) and in the selfing annual *Arabidopsis thaliana* (Berge, Nordal, and Hestmark, 1998). All these case studies are consistent with the view that a close relationship between geographic and genetic distances may only be expected if gene flow preventing isolation by distance is a simple function of geographical distance and if such gene flow is not overlaid by strong effects of genetic drift. The absence of such a correlation therefore suggests an important role for genetic drift in *R. reptans,* in line with the observed pronounced differentiation among populations.

Population size and genetic variation—There was a significant positive correlation between molecular genetic variability and population size in *R. reptans* (see Fig. 4). Old vegetation records and observations of local botanists suggest that the studied populations have existed at least for decades (Schröter and Kirchner, 1902; Baumann, 1911; Lang, 1967). The only exception is population Sipplingen, which colonized after habitat restoration several years ago (Krumscheid and Schöllhorn, 1993; M. Peintinger, personal observation). The plants of Sipplingen are most similar to one genotype from Bibershof and one from Ludwigshafen, which is only 6 km farther at the Überlingersee. Therefore we speculate that Sipplingen was colonized from Ludwigshafen by means of water drift, transport by water fowl, or human activity. Despite the potentially very long life span of the clonal *R. reptans,* genetic drift (and in the case of Sipplingen a founder effect, which is a special case of genetic drift) appears to have reduced genetic variability in small populations.

Despite much discussion about possible genetic consequences of reductions in population size and increased isolation due to habitat fragmentation (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young, Boyle, and Brown, 1996), few studies have investigated the relationship between population size and genetic variation in plants. In 11 of 16 species of perennial herbs and woody plants cited in Frankham (1996) there was a significantly positive relationship between size and at least one measure of genetic variability of populations. In smaller populations of the highly selfing short-lived monocarpic *Gentianella germanica,* molecular variance measured with RAPD was smaller than in larger ones (Fischer and Matthies, 1998a). Plants in smaller populations of the allotetraploid herb *Microseris lanceolata* had lower mean numbers of alleles than those in larger populations (Prober, Spindler, and Brown, 1998). Weak but significant positive correlations between population size and isozyme-based measures of genetic diversity were found in the clonal grass *Festuca ovina* and the perennial herb *Lychnis viscaria,* while no such correlations were found in the inbreeding annual *Arabidopsis thaliana* (Berge, Nordal, and Hestmark, 1998). No clear relationship between plant life history and the correlation between population size and genetic variation is established at present, possibly due to the still small number of studies. However, a positive correlation between the two has not only been reported for short-lived monocarpic species, but also for long-lived perennial herbs, trees, and clonal plants.

Conservation implications—The observed strong genetic differentiation among populations of *R. reptans* indicates that management for the conservation of genetic variability in *R. reptans* should not only aim to preserve large populations but also as many of the small populations as possible.

Populations of *R. reptans* covering <100 m^2 had reduced levels of genetic variability. Because in Central Europe many existing populations of *R. reptans* are <50 m^2 (Dienst and Weber, 1993), levels of genetic variability are likely to be reduced in these populations. Our finding that in only three cases ramets in 25-cm distances from each other belonged to the same clone suggests that there may be several genotypes per square metre and hundreds or even more than 1000 genotypes per 100 m^2. A population size of $\sim$1000 individuals has been predicted to be required to sustain quantitative genetic variation (Lande, 1995).

Further reduction of genetic variability by genetic drift should be avoided. Therefore management should aim to increase the number of plants in small populations. A further management measure could be artificial gene flow among populations (Oostermeijer, Altenburg, and Den Nijs, 1995). However, fitness consequences of this measure are not necessarily beneficial because previous studies have not only found increased offspring fitness after interpopulation crosses (Oostermeijer, Altenburg, Den Nijs, 1995) but also outbreeding depression after crosses among plants from different neighborhoods or populations (Price and Waser, 1979; Waser and Price, 1994; Fischer and Matthies, 1997).

Positive correlations between fitness-related characters and genetic variability have been found in a number of plant species (e.g., Linhart and Mitton, 1985; Oostermeijer et al., 1995; Fischer and Matthies, 1998a). However, the relative importance of random genetic processes for demography and extinctions of rare plants is under debate (Soulé and Mills, 1992). We observed effects of population differentiation and genetic drift on the genetic structure of *R. reptans* despite its outcrossing and clonal life history. This suggests that similar levels of

sizes and degrees of isolation of habitats would lead to even more pronounced effects on the genetic structure of shorter lived and selfing plant species. While we clearly need more studies that simultaneously relate population size, and environmental and genetic variation to plant fitness in declining plant species, our study suggests that this effort must not only be limited to short-lived selfing species.

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APPENDIX 1. Pairwise genetic distances (Φ_{st}, lower left triangle of the matrix) among 17 populations of *Ranunculus reptans.* Levels of significance are given in the upper right triangle of the matrix: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P values indicate the probability that a random genetic distance (F_{ST}) is larger than the observed distance and are based on 1000 iterations. Populations are numbered as in Table 1.

Population	Population																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		***		***		***	*	***			***	***	***		***		*
2	0.182		***	***	***		*	***	***	*	*		***	*	***	***	***
3	0.040	0.237		***		***		*	*	***	***	***	***	*	***		*
4	0.333	0.450	0.449		***	***	***	***	***	***	***	***	***	***	***	***	***
5	0.025	0.212	0.044	0.441		***				*	***	***	***		**		***
6	0.140	0.026	0.231	0.484	0.221		***	***	***		***		***	***	***	*	***
7	0.085	0.158	0.033	0.501	0.017	0.186			*	*		***	***		***	*	
8	0.145	0.295	0.076	0.569	0.073	0.316	0.065			***	***	***	***	*	***	*	***
9	−0.028	0.258	0.091	0.387	0.042	0.215	0.098	0.111		***	***	***	***	**	***	***	***
10	0.035	0.123	0.122	0.475	0.127	0.059	0.146	0.268	0.161		*	*	***	*	***	*	***
11	0.201	0.151	0.189	0.508	0.169	0.168	0.074	0.263	0.222	0.221		***	***	**	*	***	***
12	0.172	0.003	0.236	0.399	0.205	0.073	0.178	0.322	0.253	0.144	0.190		***		***	***	***
13	0.199	0.466	0.330	0.611	0.391	0.453	0.420	0.453	0.242	0.408	0.525	0.493		***	***	*	***
14	0.057	0.081	0.062	0.311	0.016	0.134	0.027	0.131	0.099	0.065	0.151	0.029	0.391		***	***	***
15	0.079	0.352	0.144	0.585	0.088	0.268	0.114	0.204	0.151	0.232	0.219	0.359	0.439	0.228			*
16	0.053	0.304	0.137	0.532	0.162	0.208	0.185	0.268	0.156	0.170	0.202	0.310	0.450	0.145	0.078		
17	0.077	0.251	0.069	0.484	0.105	0.184	0.093	0.151	0.155	0.218	0.141	0.265	0.352	0.163	0.083	0.040	

APPENDIX 2. Pairwise tests of heteroscedasticity of molecular variance among 17 populations of *Ranunculus reptans.* Bartlett's *B* is given for each pair of populations in the lower left triangle of the matrix. Levels of significance are given in the upper right triangle of the matrix: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *P* values indicate the probability that a random *B* is larger than the observed *B* and are based on 1000 iterations. Populations are numbered as in Table 1.

Population	Population																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		***		***		***	*	***			***	***	***		*		
2	2.722		***	***	***		*	***	***	**	*		***	***	***	***	**
3	1.332	2.978		***		***			*	*	***	***	***		***		
4	6.655	6.934	7.555		***	***	***	***	***	***	***	***	***	***	***	***	***
5	1.425	2.511	1.240	6.390		***				**	*	***	***		*		*
6	2.175	1.117	2.854	7.904	2.612		***	***	***		***	*	***	***	***	***	***
7	2.065	2.151	1.267	7.708	1.019	2.470			*	***		***	***		*	*	*
8	2.431	3.335	1.556	8.468	1.365	3.619	1.333		*	***	***	***	***	***	***	*	***
9	0.835	3.252	1.587	6.781	1.267	2.722	1.833	1.880		***	***	***	***	***	***	***	***
10	1.209	1.882	1.760	7.673	1.809	1.329	2.127	3.075	2.068		***	***	***		***	***	***
11	3.158	2.076	2.617	6.967	2.141	2.337	1.445	2.867	3.004	2.834		***	***	**	***	*	**
12	2.607	0.923	2.963	6.145	2.444	1.463	2.344	3.632	3.190	2.038	2.415		***		***	***	***
13	3.819	6.180	4.607	9.608	4.747	6.180	5.370	5.468	3.754	5.413	6.533	6.667		***	***	***	***
14	1.427	1.602	1.355	5.608	1.064	1.960	1.249	1.991	1.652	1.337	2.333	1.195	5.448		***	*	***
15	2.228	4.275	2.267	9.331	1.562	3.382	1.787	2.447	2.420	2.998	2.586	4.366	5.569	3.088			*
16	1.014	2.202	1.320	4.960	1.433	1.653	1.621	1.972	1.397	1.417	1.758	2.233	3.531	1.375	1.242		
17	1.653	3.093	1.410	8.015	1.655	2.384	1.684	2.080	2.145	2.587	2.149	3.237	4.774	2.207	1.694	0.956	