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Effects of a snowmelt gradient on the population structure of *Ranunculus alpestris* (Ranunculaceae)

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Abstract

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An analysis of population structure and genetic differentiation was conducted in three populations of the outbreeding perennial *Ranunculus alpestris* L. The populations were located along environmental gradients caused by gradual melting of snow (Pizol mountains, Switzerland). In each population, plants were collected along two transects that were perpendicular to the snowmelt line. At the time of collection, flowering stage was highly correlated with the position of the plants along the transects. However, these differences in flowering time among individuals appeared to be controlled entirely by the environment, as they did not persist in the greenhouse. A regression model based on phenology data from plants grown in the greenhouse suggested that in most transects there should be no overlap in flowering time between the earliest and latest flowering individuals. However, based on three polymorphic allozyme loci genetic differentiation between subpopulations along the transects was low (F_{ST} = 0.022). Hardy-Weinberg equilibrium tests indicated that random mating existed at a subpopulation level within populations, but not among the three sampled populations. By comparison of the genetic differences among subpopulations along snowmelt gradients with those between populations, we showed that the effect of the snowmelt gradient on genetic differentiation was weak and that isolation by distance may be more important for structuring the populations of *R. alpestris*.

Key words: Allozyme, environmental gradient, phenology, population biology, *Ranunculus alpestris*.

Introduction

Plants may respond to a variable environment by the evolution of sufficient phenotypic plasticity (Levin and Kerster 1968) or by genetic adaptation to particular con-

ditions (Stanton et al. 1997). Genetic differentiation among subpopulations is likely to take place where environmental patch differences are consistent over time and where most seed and pollen are locally dispersed within patches (Wright 1969). In such, at least partially isolated subpopulations, two processes promote genetic differentiation: genetic drift and/or localised natural selection (Wright 1943). Accordingly, even large, continuously distributed populations can exhibit genetic structure due to restricted gene flow, i.e. isolation by distance (Wright 1978). The genetic variation of corresponding subpopulations may thus depart from that expected in a random-mating population (Turner et al. 1982).

Because of the many different interacting processes that may generate genetic structure within populations (Grosberg 1991), the study of particular environmental situations that are expected to attenuate the effect of only one of these processes can be informative. For example, differences in flowering time among individuals, which restrict the movement of pollen should enhance genetic structure and facilitate localised adaptation in patchy environments (Cruzan et al. 1994). Such local differences in flowering time are obvious in the alpine flora due to the effects of snowmelt time, which is strongly influenced by topography and winter snow deposition (Billings and Bliss 1959, Kudo 1991). Accordingly, microsites separated by relatively short distances may be inhabited by plants whose phenologies differ by several weeks (Stanton et al. 1994).

The present study aims at a better understanding of the potential effect of snowmelt time on the genetic structure of populations, a topic that has received little attention until recently (Stanton et al. 1994, 1997). We describe the genetic and phenological structure of three populations of *Ranunculus alpestris* L. (Ranunculaceae) in steep snowmelt gradients and address the following questions: (1) Is the snowmelt gradient correlated with differences in flowering time? (2) Do the differences observed in situ persist in greenhouse conditions; e.g., are they genetically fixed? (3) Do differences in flowering time across snowmelt gradients affect genetic differentiation of subpopulations? Genotype frequencies at neutral allozyme loci calculated for different populations and subpopulations should provide an answer to this latter question.

Materials and Methods

The study plant

Ranunculus alpestris is a subalpine or alpine perennial (Landolt 2003) that reaches 20 cm in height. It prefers open, moist habitats such as grasslands, screefields and snowbeds. It endures long snow cover and is mostly found on calcareous substrate. The species is diploid and self-incompatible (Müller and Baltisberger 1983). Adult plants produce tightly clustered ramets, but no real vegetative spread occurs. *Ranunculus alpestris* produces showy white flowers between June and August. Gravity and snowmelt water are probably the main vectors of seed dispersal, but, according to Müller-Schneider (1986), epichory also occurs in this species. Distances of seed dispersal are unknown for *R. alpestris*, but a range of only 0.1 to 1 m has been found in another alpine buttercup species, *R. adoneus* L. (Scherff et al. 1994).

Sampling

Three populations of *R. alpestris* were studied on meadows bordering a ground depression in the Pizol mountains of north-eastern Switzerland. The populations were

separated from each other by several hundred meters and were located on a south-facing slope at an altitude of about 2230 m. A total of 269 living individuals was collected in late summer 2000 along six transects (two transects per population). The transect lengths ranged between 4.5 and 14 m, depending on the spatial expansion of the local *R. alpestris* population (Fig. 1). Transects were layed perpendicular to the snowmelt line. Along the transects, the exact locations of all individual plants were recorded as well as their phenological stage. The flowering stage was described by five categories: (1) "flower bud", (2) "young flower", (3) "old flower", (4) "faded flower" and (5) "fruits". If a plant did not produce flowers, it was given the stage of its nearest neighbour. The collected plants were individually potted into clay soil and kept in a greenhouse located at the Hönggerberg campus of ETH Zürich, where 94.1% of the plants survived.

Phenology

The phenology of each plant kept in the greenhouse during the following year was recorded every week from week 14 (beginning of April) until week 25 (end of June). As in the field, five flowering stages were distinguished (see above), but stages 4 and 5 were later combined into a single category. Flowers in stages 2 and 3 were assumed to be receptive

To test whether snowmelt time has an effect on the flowering time of the plants, we calculated Spearman nonparametric correlation coefficients between the position of each plant along the transect and its phenological stage, both under natural conditions and in the greenhouse using JMP version 4.0 (SAS). In order to estimate the maximum duration of flowering of each plant and possible overlap in flowering time along the transects, we constructed a regression model. Parameters used were the flowering stages recorded along the transects on 21 July 2000 and the estimated duration of each flowering stage based on the phenology data collected in the greenhouse.

Allozyme analysis

Fresh mature leaves were collected from the greenhouse plants twelve hours prior to enzyme extraction and stored overnight in Eppendorf tubes at 4°C. After having been frozen in liquid nitrogen, tissues were ground for 3 min using a bead mill (Retsch MM2000). Afterwards, 170 µl of extraction buffer (at 4°C) was added and the samples were again shaken for three minutes. The extraction buffer as modified from Soltis et al. (1983) contained 100 mM Tris-HCl buffer at pH 7.5, 1.3 mM EDTA tetrasodium dihydrate, 11.5 mM potassium chloride, 10 mM magnesium chloride hexahydrate, 10% (v/w) polyvinylpyrrolidone (PVP–40) and 0.1% (v/v) 2-mercaptoethanol. Subsequently, the remaining plant tissue was removed by centrifugation at 14000 rpm for 10 min. Horizontal starch (12.8% w/v) gel electrophoresis was performed as described by Soltis et al. (1983) and Huber and Leuchtmann (1992) using buffer system 5 at pH 7.2. Gels were stained according to the recipes of Soltis et al. (1983) and Wendel and Weeden (1989).

A total of 22 enzyme systems was tested. Among these, seven were monomorphic, two showed no reaction and ten were difficult to interpret for various reasons and therefore discarded from analysis. Only three enzyme systems were kept for further analysis: esterase EST (Enzyme Commission Number 3.1.1.–; IUB 1984), isocitrat dehydrogenase IDH (1.1.1.42) and shikimate dehydrogenase SKDH (1.1.1.25), each with one interpretable locus.

Jean-David Gerber et al.

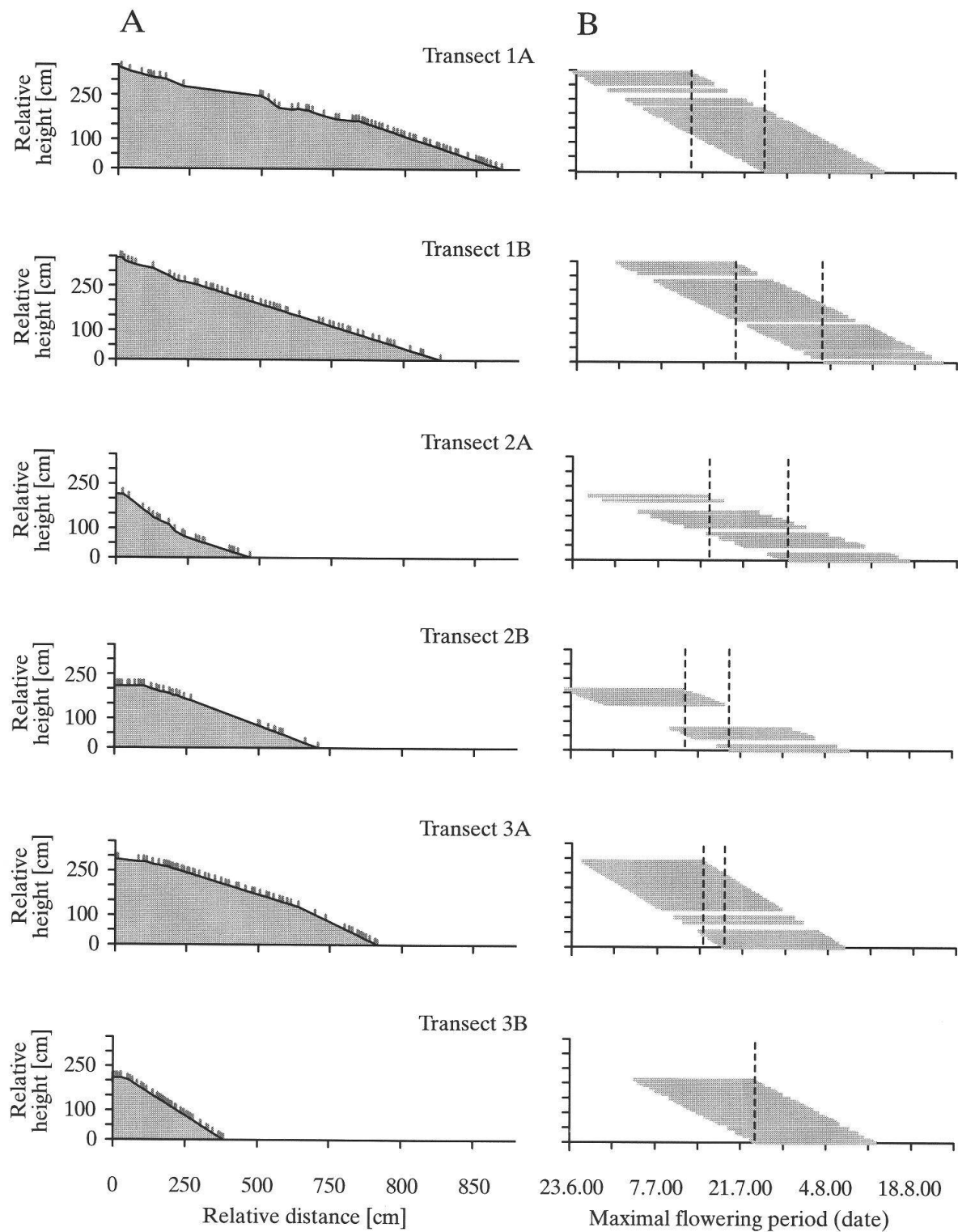Fig. 1. A: Transect profiles; a total of 269 individuals of *Ranunculus alpestris* was collected along six transects; marks along transects correspond to the location of plants. B: Maximum duration of flowering along the transects according to a regression model; vertical broken lines indicate the time periods of no overlap in flowering between plants growing at the bottom and top of the transects.
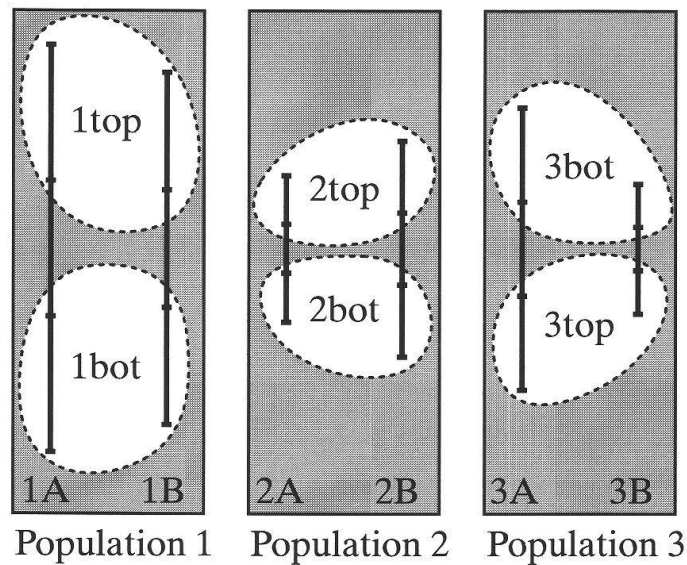
Fig. 2. Hierarchical structure of the populations of *Ranunculus alpestris* studied with five grouping levels: total data set, population (grey rectangles), transect (black lines; e.g., 1A and 1B), grouped subpopulation (dashed lines; e.g., 1top and 1bot) and subpopulation level (upper and lower thirds of transect lines; e.g., 1Atop and 1Abot).

Statistical analyses

We distinguished three grouping levels (Fig. 2). (1) The total data set (T) consisting of all individuals sampled; (2) the population level (R) each with two transects; (3) the subpopulation level (S). To define subpopulations (S), we divided each transect into individuals growing in the upper third of the transect, in the middle third (transition zone) and in the lower third. Individuals from the transition zone were discarded to obtain spacially distinct subpopulations. For comparison, one more level was defined: (4) Grouped subpopulations (gS) per population, where the data from the two corresponding transects were merged, again leaving out those individuals from the middle third of the transects. Sample sizes used in analyses of populations, transects and subpopulations are indicated in table 1.

To test for Hardy-Weinberg equilibrium (HWE), we applied a global HWE-test using GENEPOP (http://wbiomed.curtin.edu.au/genepop). Fixation indices F_{SR}, F_{RT} and F_{ST} and the inbreeding coefficient F_{IS} were calculated with the formulas of Weir and Cockerham (1984), again using GENEPOP. F_{IS}-values were calculated at each hierarchical level (T, R, gS and S) and their averages given as mean F_{IS} per enzyme and mean F_{IS} over three enzymes (Tab. 1). To test for significance of genetic differentiation among populations, transects and subpopulations, we made pairwise comparisons using Fisher exact tests per enzyme in GENEPOP as described by Raymond and Rousset (1995). We then used Fisher's method (Sokal and Rohlf 1997) to combine the P-values obtained per enzyme. In addition, we carried out a principal component analysis (PCA) of the distribution of alleles at different hierarchical levels using JMP version 4.0 (SAS).

Tab. 1. Inbreeding coefficients F_{IS} in *Ranunculus alpestris* according to Weir and Cockerham (1984) at different hierarchical levels (Fig. 2).

Level	Number of plants	Allozyme			Mean total F_{IS}
		EST	IDH	SKDH	
Total data set (T)					
	253	0.152	0.030	0.206	0.102
Populations (R)					
1	112	0.266	−0.045	0.036	
2	50	0.057	0.123	0.306	
3	91	−0.010	0.060	0.305	
Mean		0.123	0.026	0.198	0.089
Transects (T)					
1A	59	0.238	−0.158	0.198	
1B	53	0.297	0.090	−0.130	
2A	20	0.363	0.008	−0.027	
2B	30	−0.128	0.165	0.253	
3A	54	−0.146	0.187	0.347	
3B	37	0.108	−0.213	0.265	
Mean		0.122	0.017	0.172	0.079
Grouped subpopulations (gS)					
1top	38	0.430	0.026	−0.104	
1bot	38	0.028	−0.231	−0.072	
2top	17	−0.248	0.227	0.299	
2bot	17	0.158	0.053	−0.071	
3top	31	−0.014	0.169	−0.074	
3bot	31	0.182	−0.042	0.275	
Mean		0.107	0.001	0.066	0.045
Subpopulations (S)					
1Atop	20	0.278	−0.009	−0.086	
1Abot	20	0.209	−0.329	−0.027	
1Btop	18	0.505	0.017	−0.133	
1Bbot	18	−0.178	−0.139	−0.062	
2Atop	7	−	0.273	−	
2Abot	7	0.727	−0.304	−	
2Btop	10	−0.455	0.122	0.386	
2Bbot	10	0.100	−0.050	0.407	
3Atop	18	−0.071	0.139	−0.097	
3Abot	18	−0.123	0.026	−	
3Btop	13	−0.019	−0.222	0.645	
3Bbot	13	0.585	−0.165	0.283	
Mean		0.131	−0.063	0.141	0.029

Results

Phenology

For *in situ* phenology data, a Spearman coefficient indicated highly significant correlation between flowering time and position along the transect ($P < 0.001$). In contrast, no such correlation was detected for the plants growing in the greenhouse, apart from one case, transect 3B. In this transect, however, the number of flowering individuals was very low ($N = 4$) and the resulting correlation probably accidental.

Based on a regression model using flowering stages recorded along the transects in the field and phenology data collected in the greenhouse, we obtained the following approximations for the duration of each stage: flower bud – seven days , young flower – ten days, old flower – ten days, faded flower and fruits – 20 days. If the flowering time lasts 20 days as assumed in our regression model (young and old flower combined), we can expect a potential overlap in flowering time among all individuals only in transect 3B, while individuals at the bottom and top of transects 1A, 1B, 2A and 2B should be separated (Fig. 1).

Allozymes

The enzyme systems EST, IDH and SKDH were found to be variable in *R. alpestris* and could be reliably scored. EST is a monomer or a dimer with up to ten loci (Tanksley and Rick 1980, Wehling and Schmidt-Stohn 1984). We mostly obtained two loci and the cathodal one could be scored. It defined a monomer with three alleles. IDH is a cytosolic dimer with one locus (Ni et al. 1987, Tanksley 1984). We found three alleles at this locus.

In addition there was a second faster moving active zone consistently present, which was not scored. The monomeric SKDH can be coded by one locus or two loci (Weeden and Gottlieb 1980). We found a locus with two alleles and several additional, but less distinct bands, which were not scored.

Genetic variation and differentiation

Hardy-Weinberg tests were conducted at the population, the transect, the grouped subpopulation and the subpopulation level (Tab. 2, Fig. 2). All three populations showed a significant deviation from HWE ($P < 0.05$), while only two transects (1B and 3A) significantly deviated from HWE ($P < 0.05$). Transect 3B, where pollen exchange between individuals growing at both extremities seemed to be possible (see above), showed no significant deviation from HWE. Among subpopulations – grouped or not – none except one (1top) significantly deviated from HWE (Tab. 2).

The principal component analysis (PCA) of allele distribution illustrates genetic differences at different hierarchical levels (Fig. 3). Pairwise comparisons using differentiation tests (Fisher exact tests) after Raymond and Rousset (1995) indicated that populations 1 and 2, and 2 and 3 were significantly different from one another ($P < 0.001$), while populations 1 and 3 were not ($P = 0.104$; Fig. 3). The two transects of population 1 did not show any significant differentiation, whereas the transects of populations 2 and 3 were significantly different ($P < 0.01$), respectively. No significant differentiation could be found among grouped subpopulations, and only subpopulations 1Btop and 1Bbot of transect 1B differed significantly ($P < 0.05$). However, two pairs of the grouped subpopulations differed significantly from each other (2Atop from 2Btop and 3Abot from 3Bbot at $P < 0.01$ and $P < 0.05$, respectively).

Tab. 2.   Hardy-Weinberg-Equilibrium-tests after Rousset and Raymond (1995) for heterozygote deficiency at the population, transect, grouped subpopulation and subpopulation level in *Ranunculus alpestris*. * = P < 0.05.

Group	P-Value	Group	P-Value	Group	P-Value
Populations					
1	0.019*	2	0.025*	3	0.053*
Transects					
1A	0.126	2A	0.166	3A	0.054*
1B	0.026*	2B	0.138	3B	0.476
Grouped subpopulations					
1top	0.026*	2top	0.223	3top	0.410
1bot	0.953	2bot	0.290	3bot	0.135
Subpopulations					
1Atop	0.314	2Atop	0.234	3Atop	0.493
1Abot	0.822	2Abot	0.271	3Abot	0.539
1Btop	0.088	2Btop	0.465	3Btop	0.388
1Bbot	0.921	2Bbot	0.304	3Bbot	0.166

Mean fixation indices over all three allozymes studied decreased steadily along the structural hierarchy: $F_{IS} = 0.102$ at the total data set level, $F_{IS} = 0.089$ in populations, $F_{IS} = 0.045$ in grouped subpopulations and $F_{IS} = 0.029$ at the subpopulation level (Tab. 1). This indicates that the average heterozygote deficiency decreases at the smaller spatial scales. The mean F_{IS} values per enzyme system presented a particular pattern: while F_{IS} values of IDH were around zero at all hierarchical levels, the F_{IS} values of EST and SKDH were consistently higher (> 0.1 in all but one case). The F_{IS} values of EST and SKDH also decreased from total data set to the grouped subpopulation level similar to the mean F_{IS} values over all populations (Tab. 1). However, they increased again at the subpopulation level. Some of the F_{IS} of these enzyme systems were very high, attaining a maximum of $F_{IS} = 0.727$ at EST in subpopulation 2Abot (Tab. 1).

The amount of differentiation among subpopulations (F_{ST}) was higher for the enzymes EST and SKDH ($F_{ST} = 0.031$ and $F_{ST} = 0.040$, respectively) than for IDH ($F_{ST} = 0.010$; Tab. 3). The average F_{ST} over all three loci was 0.022, which indicates weak genetic differentiation of the subpopulations. It is apparent that, on average, genetic differentiation mostly occurred between subpopulations and populations ($F_{RT} = 0.025$, $F_{ST} = 0.022$; Tab. 3) but not between subpopulations within populations.

Discussion

The primary goal of this study was to determine whether flowering time differences affect genetic differentiation within three populations of *Ranunculus alpestris*. Our assumption was that gradual snowmelt within a site leads to a shift in the flowering
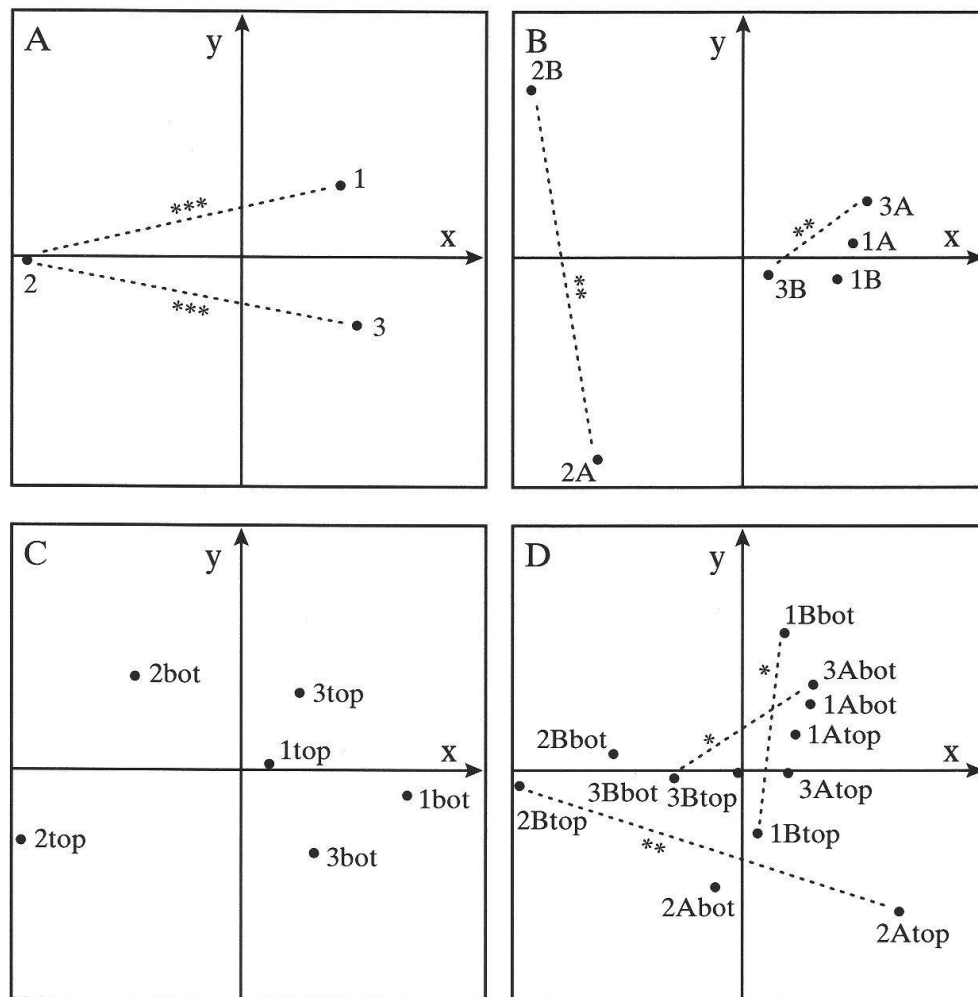
Fig. 3. Principal component analysis (PCA) of allele distribution at different hierarchical levels in *Ranunculus alpestris*. A: Three populations (x-axis explains 87.6% and y-axis 12.5% of the variance). B: six transects (x-axis: 45.0%, y-axis: 31.8%). C: six grouped subpopulations (x-axis: 58.9%, y-axis: 16.7%). D: twelve subpopulations (x-axis: 46.5%, y-axis: 28.9%). Pairwise comparisons are based on Fisher exact tests: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

time of plants. This should impede effective pollen flow across the snowmelt gradient and inforce genetic differentiation between subpopulations at early- and late-melting sites due to selection and/or genetic drift. The genetic analyses described here, however, suggest that the genetic differentiation between subpopulations along a snowmelt gradient was marginal and, thus, did not support this hypothesis.

Phenology

Based on a regression model, we tested whether there was a correlation between phenological state of plants and snowmelt timing growing along transects. At natural sites at the time of plant collection in late summer phenological state and snowmelt time were clearly correlated. In contrast, with the same plants grown in the greenhouse we observed no correlation between these two parameters in the following year. This

Tab. 3.   Fixation indices F_{SR} (subpopulation/population), F_{RT} (population/total data set) and F_{ST} (subpopulation/total data set) in *Ranunculus alpestris* according to Weir and Cockerham (1984). [a] = negative values are mathematical artefacts.

Allozyme	F_{SR}	F_{RT}	F_{ST}
EST	−0.021[a]	0.051	0.031
IDH	0.012	0.006	0.010
SKDH	0.025	0.016	0.040
Mean	−0.003	0.025	0.022

suggests that flowering time of *R. alpestris* is controlled by environmental conditions, i. e. snowmelt timing, and not by genetic factors. Stanton and Galen (1997) obtained similar results for *R. adoneus* using transplantation experiments, in which individuals were moved along an environmental gradient.

Assuming a flowering time of 20 days on average in *R. alpestris* plants, only the relatively long and steep transects 1A, 1B, 2A and 2B exhibited non-overlapping flowering periods of individuals at opposite ends of ecological gradients, while in the two transects 3A and 3B pollen exchange should have principally been possible across the entire gradient. Since spatial patterns of snow deposition tend to be consistent among years (Kudo 1991), the relative flowering patterns should also remain constant among years. If, however, flowering duration would be longer than 20 days and/or more variable among plants, which seems to be likely at higher elevations, an even greater overlap in flowering phenology would occur.

Genetic variation and differentiation

Following our hypothesis of limited gene flow along the snowmelt gradient among subpopulations, we expected a significant departure from the Hardy-Weinberg equilibrium (HWE), both at the population and the transect levels (P, Tr). Our results partially confirmed this expectation; all three populations deviated from HWE, but four of six transects did not. In contrast, all subpopulations (except one) were in HWE. We conclude that random mating did not take place among populations and within some of the transects, because they were actually divided into subpopulations.

Did genetic differentiation occur along the snowmelt gradient investigated? If the answer is yes, we would expect that subpopulations differ within transects but not within grouped subpopulations. None of the grouped subpopulations differed significantly from one another within a population. Within transects, subpopulations 1Btop and 1Bbot, and among the grouped subpopulations 2Atop and 2Btop, and 3Abot and 3Bbot differed significantly from each other, partially confirming our expectations. The observed differences among subpopulations with the same flowering time (two cases out of six) and the weak differentiation between subpopulations along the snowmelt gradient (one case out of six) suggested that isolation by distance may have a stronger structuring effect on the three populations studied than snowmelt timing.

To infer non-panmictic groups in the three populations of *R. alpestris*, we calculated F_{IS} values at different hierarchical levels, expecting them to decrease when progressing from the whole data set to subpopulation level (Wahlund effect; Hartl and Clark 1997). Indeed, our results met these expectations with the average inbreeding

coefficients decreasing from 0.102 at the highest hierarchical level to 0.029 at the lowest level. Moreover, average F_{IS} values differed among loci; this value was about zero at all hierarchical levels for IDH, but consistently higher than 0.1 for EST and SKDH. Interestingly, while the F_{IS} values of EST and SKDH decreased from the total data set level (T) to the grouped subpopulation level (gS), they increased again at the subpopulation level (S). However, the latter could be caused by calculation errors and could thus just be artefacts. In summary, the fixation indices gave a less clear answer to our principal hypothesis, but they confirmed that most of the genetic variation was found within subpopulations of *R. alpestris*.

Conclusions

Differences in flowering time of *Ranunculus alpestris* observed in the field were clearly correlated with the snowmelt gradient. However, the greenhouse experiment indicated that these differences did not have a genetic basis, but were mainly induced by environmental conditions. Although we found some differences in allele frequencies between individuals at early- and late-melting sites, which could be attributed to differences in flowering time, our results gave more direct evidence for isolation by distance as a mechanism for differentiation. Gene flow between early- and late-melting sites can either take place by pollen exchange or seed dispersal (Ouborg et al. 1999). At our sites, the latter may be more important, because pollen exchange seemed to be limited due to differences in flowering time.

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