Genetic diversity and differentiation of alpine plant species in Switzerland assessed with amplified fragment length polymorphism (AFLP)

Diploma thesis of Stefan Weber

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Abstract

Biodiversity can be measured at different levels. While it is mostly species richness and habitat variation that are taken into account, genetic diversity contributes a further dimension to the biodiversity. Genetic diversity has not been measured on a regular grid until now. The EU project IntraBioDiv investigates correlations of three levels of biodiversity of alpine plant species. Genetic diversity is assessed using a regular sampling grid with cells of 25x23 km. In the current study, a down-scaling approach was used to test reliability of genetic diversity measures on two different spatial scales. Four grid cells in the Swiss Alps were divided into four subcells. DNA from ten common alpine plant species was analyzed using amplified fragment length polymorphism (AFLP). Nei's average gene diversity generally yielded a good correlation of the values obtained on the large scale and the small scale but some species showed a different distribution. Taking all species together, the distribution of diversity was different between the two spatial scales. This indicates that we should be careful by comparing genetic diversity measures of two spatial scales.

Genetic diversity was different among the single species. Wind-pollinated species tended to have higher diversity values than the other species. Substrate seemed not to have an effect on the absolute amount of diversity, which was rather dependent on the laboratory where the AFLP procedure has been performed.

It is not only diversity within populations that describes the genetic structure of populations but also genetic differentiation. F_{ST}-values of each species were calculated. The majority of variation lay within populations. Only *Arabis alpina* showed more variation among populations than within populations. This species showed a partition into two groups by analyzing the population structure but these groups where not distributed with a clear pattern. *Carex sempervirens*, *Geum reptans* and *Rhododendron ferrugineum* showed a distribution into an eastern and a western group. This separation is congruent with biogeographic lines present in eastern Switzerland. The other species were not differentiated into two groups.

Introduction

Biodiversity can be measured at different levels. The most widely used aspect is species diversity. A huge amount of books on regional, national or international floras are available. Species richness is a component of diversity that can be observed directly and people began to measure it a long time ago. Also an important part of biodiversity is the variety of the landscape which affects habitat diversity. It is known that the substrate influences the amount of species richness in alpine plant species (Wohlgemuth, 2002). Therefore, assessing habitat diversity may allow for prediction of species richness in a region. A third component of biodiversity is the genetic diversity. It is important for a species that the genetic information is variable between individuals in order to prevent negative effects due to a too small amount of genetic variability in a population or to adapt to changing environmental conditions. In other words, the amount of genetic variability of a species will determine its adaptive capacity and evolutionary potential (Till-Bottraud, Gaudeul, 2002). It has been shown that the persistence of populations may be positively linked to genetic variability (Frankham, Ralls, 1998). This finding is supported by Newman & Pilson (1997) who found that the extinction rate of a wild plant was higher in experimental populations with low versus high genetic variation when both were planted in the field. All these studies demonstrate that the genetic diversity of a species may play an important role in preserving the fitness of a species for future challenges.

Alpine as well as arctic and subarctic species are expected to suffer from the climatic changes in the last decades. One possibility for the plants in the Alps is to shift their distribution area onto higher elevations (Grabherr *et al*., 1994). However, this strategy is limited by the height of the mountains. Once arrived on the top of the mountains, no further ascension is possible. Therefore, plants that are able to adapt to the ongoing climatic warming may have a competition advantage over their non-adapted competitors.

Different spatial scales can be used to investigate diversity at any of the three levels of biodiversity mentioned above. One possibility to assess diversity is to use a regular grid

and to determine diversity within each grid cell. This has frequently been done to estimate the number of species in a particular area. There is a huge amount of literature describing species richness on different spatial scales, from local estimations to global approaches. Willis & Whittaker (2002) give a short overview of studies that investigated species richness on different spatial scales. These studies showed different patterns of species diversity due to varying environmental factors which act on different spatial scales. This shows us that the observation on a particular scale cannot be generalized but merely applied to the scale studied. A small scale may give a better resolution for a local problem, whereas a large scale would be better to assess landscape patterns or processes. That means that we do not know whether species diversity assessed on a particular scale is also relevant on larger or smaller scales.

The same scale problem appears in the investigations of genetic diversity within species. There are several possibilities to assess genetic diversity of a particular plant species. Rivera-Ocasio *et al*. (2006) investigated the genetic structure and diversity of a tropical wetland tree on three different scales (<10km, <100km and >1000km). At all three spatial scales, most of the genetic variation occurred within populations, but as the spatial scale increased, there was an increase in the among-population variation. Therefore, it is crucial what spatial scale we choose to infer genetic diversity of a species. If the scale is too small, chance events will affect diversity estimates (Ward, 2006). On larger scales, we are not able to detect coherences which are relevant on smaller scales. Therefore, to estimate genetic diversity of plant species, we have to choose a scale on which we will acquire an appropriate measure of diversity. Mostly, genetic diversity is measured not on a regular grid, but rather taking into account several populations more or less equally distributed over a particular area.

In the European project IntraBioDiv (http://intrabiodiv.vitamib.com), the correlation between habitat diversity, species richness and genetic diversity is studied in high-mountain plants. For this purpose, a regular grid is laid over the entire Alps and the Carpathians, with grid cells of 25x23 km. To assess genetic diversity, plant material of 27 alpine plant species was sampled in every second grid cell from three individuals per species. This gives an estimate of plant genetic diversity on a large scale over the entire Alps. In the present study, I examined whether genetic diversity of this large grid is also

relevant for a smaller scale taking into account ten out of the 27 species of the EU project. Such down-scaling has often been performed but mostly on the species diversity level. Since no study has been done on the genetic diversity of species on a regular sampling grid, we do not know how representative genetic diversity measures of a large grid are to those on a smaller scale.

There is a large amount of methods that can be used to analyze DNA of an organism. A widespread method to investigate genetic diversity is amplified fragment length polymorphism (AFLP; Vos *et al*., (1995)). AFLPs can be used to investigate diversity within populations or differentiation among populations. With the AFLP method we investigate only a random part of the total DNA, and we do not know if the fragments amplified are from the nuclear, chloroplast or mitochondrial genome. AFLP provides a good method to measure genetic diversity. A huge amount of literature could be listed which deals with genetic diversity obtained with AFLPs, showing that this method is very common in scientific literature.
Genetic variation is dependent on life-history traits (Hamrick, Godt, 1996; Nybom, 2004). Namely the breeding system, seed dispersal mechanism, life form and also the geographic range explain the distribution of the genetic diversity of species.

However, it is not only diversity that is a relevant component of the genetic structure of a species, but also the genetic differentiation. It is known that species can be differentiated due to low gene flow between spatially distant populations (Pluess, Stöcklin, 2004). Other aspects are historical processes that might have influenced genetic differentiation observed at present. An important process is the reimmigration of plant species into the Alps after the last ice age. Schönswetter *et al*. (2005) detected with molecular markers potential glacial refugia where alpine plant species might have survived the last ice age. The plant species might have immigrated from those refugia into their current alpine habitats. One may expect genetic difference between those populations coming from different refugia. There are also recent and on-going processes that can influence genetic differentiation. It is known that habitat fragmentation can have population genetic consequences (Young *et al*., 1996). One can imagine that different species react

differently to these processes. Therefore, the genetic differentiation would be different between the study species.

In this study, I intended to detect genetic diversity of ten common alpine plant species in the Swiss Alps using a regular grid system which is four times smaller than the one of the EU project IntraBioDiv. I examined whether genetic diversity of the large grid is also relevant for the small scale. In order to do a down-scaling analysis, I chose four of the grid cells from the EU project IntraBioDiv in the Swiss Alps and divided them into four subcells. I investigated correlations of diversity measures obtained on two different spatial scales.

Since genetic diversity is determined by environmental factors as well as species specific traits, I tested if genetic diversity measures were correlated with life-history traits of the study species. Is there a correlation of the genetic diversity and the pollen dispersal strategy? Is the genetic diversity influenced by the substrate demand of the single species? Since I included species with different traits in my analysis, I expected a different distribution of genetic diversity between the study species.

I calculated genetic differentiation for all ten study species from populations sampled in the subcells. Do they show similar population structures? And are those results in congruence with those obtained on the large scale? I consequently combined my data set of *Geum reptans* with that of Thiel-Egenter *et al*. (in prep.), which included populations of the whole range of the Alps, to test if the results obtained from populations in Switzerland fit well into those obtained from over the entire Alps.

Materials and Methods

Plant species and sampling design

Ten alpine plant species were used to assess genetic diversity within and genetic differentiation between populations. I included species which grow on different bedrock types to avoid biased results due to the substrate. Species that grow on acidic soils are *Hypochoeris uniflora, Juncus trifidus* and *Rhododendron ferrugineum*. *Arabis alpina, Dryas octopetala* and *Gypsophila repens* require calcareous substrates. *Geum reptans* and *Loiseleuria procumbens* prefer silicate but can also grow on mixed substrates. *Carex sempervirens* mainly grows on calcareous substrates but sometimes occurs on silicate. *Geum montanum* grows in meadows dominated by acidophilous species. During summer 2005, I collected individuals of these ten species in 16 regions in the Swiss Alps. The particular regions lie in the Valais, in the Canton of Uri, in the region of San Bernardino (Adula) and in the Swiss National Park (Fig. 1). The exact location of those regions is given by the sampling grid of the EU project IntraBioDiv. I chose four of these areas and divided them into four subcells (Fig. 1) obtaining 16 study sites. The subcells are defined by their geographical coordinates and have a size of about 12x12km. I chose this arrangement of the study cells to perform a down-scaling approach. Moreover, the four regions correspond to either the Swiss National Park or regions of potential new national parks (www.pronatura.ch). I only sampled populations located above 2000 meters of elevation.

In each subcell, plant material of three individuals per species was sampled along a horizontal transect with a distance of ten meters between each individual. These individuals were supposed to belong to one population. Sampled plant material was stored in silicagel. As a taxonomic control, one herbarium specimen per species was collected in each site and is now stored in the herbarium Z of the Institute of Systematic Botany, University of Zurich.

DNA extraction and AFLP protocol

Ten milligram dried plant material was used to extract DNA. For this procedure, I worked with the DNeasy 96 Plant Mini Kit by Qiagen (Hilden, Germany) following their protocol. At the end of the extraction, DNA was eluted with 200µl AE buffer by Qiagen. DNA extracts were visually checked on a 1% agarose gel. Measurements of the optical density revealed an average DNA concentration of 40ng/µl. Thus, taking 5µl of the DNA solution, I used 200ng DNA for the restriction. The restriction with EcoRI and MseI restriction enzymes was performed in an oven at 37°C for two hours (Table 1). DNA was ligated to double-stranded adaptors for two hours at 37°C (Table 2). I used EcoRI adaptors with the sequences 5'-CTC GTA GAC TGC GTA CC-3' for the forward strand and 5'-AAT TGG TAC GCA GTC TAC-3' for the reverse strand. The MseI adaptors had the sequences 5'-GAC GAT GAG TCC TGA G-3' and 5'-TAC TCA GGA CTC AT-3', respectively. DNA was amplified in two steps. The preselective polymerase chain reaction (PCR) (Tables 3, 4) was conducted with two primers consisting of a part of the adaptor sequence and the restriction site plus one selective base. The primers had the following sequences: EcoRI primer 5'-GAC TGC GTA CCA ATT CA-3' and MseI primer 5'-GAT GAG TCC TGA GTA AC-3'. A selective PCR followed the first amplification process with primers of one or two additional selective bases (Tables 5-7). The forward primers were fluorescently labeled. The PCR products were put on a 2% agarose gel to check for successful amplification. To visualize the amplified fragments, 1µl of the PCR product was mixed with 9.7µl HiDi formamide (Applied Biosystems, Foster City, USA) and 0.3µl Rox500 size standard (Applied Biosystems) and analyzed on a 3100-Avant Genetic Analyzer (Applied Biosystems).

I performed extractions of all ten species and AFLP procedure for *Geum reptans*, *Geum montanum*, *Gypsophila repens*, *C. sempervirens* and *J. trifidus* in the lab at the WSL. AFLPs for *A. alpina, D. octopetala, H. uniflora, L. procumbens* and *R. ferrugineum* were carried out at the Laboratoire d'Ecologie Alpine at the University of Grenoble by Ludovic Gielly.

Data analysis

Electropherograms of all ten species were analyzed with the software GeneMapper v3.7 (Applied Biosystems). I scored all fragments of a length between 50 and 500 bp but took only unambiguous peaks into account for further analysis. For at least two samples per species, the DNA extraction and the AFLPs were performed twice on the same plate to check for repeatability of the data. The final set of reproducible markers was transformed into a presence−absence matrix. I calculated the percentage of polymorphic loci, Nei's genetic diversity index (Nei, 1973) and the 95% confidence interval over all ten study species for the down-scaling approach. To compare genetic diversities of the particular subcells, I calculated the weighted sum of deviances from the mean average gene diversity per species for each subcell. These deviances were calculated as follows: I calculated mean average gene diversity of each species over all study cells. In each subcell, I calculated the deviance of the average gene diversity of each species from the mean value of the respective species. Finally, I summed up all deviances per subcell and divided them by the number of species occurring in the particular subcells.
Structure software version 2.0 (Pritchard *et al*., 2000) was used to infer population structure. For *Geum reptans, C. sempervirens*, *R. ferrugineum* and *A. alpina* the geographic distribution of the groups obtained with *Structure* software (Pritchard *et al*., 2000) was visualized in ArcView GIS 3.3. I further calculated F_{ST}-values for all species using the framework of an analysis of molecular variance (AMOVA) with the software Arlequin2000 (Schneider *et al*., 2000).

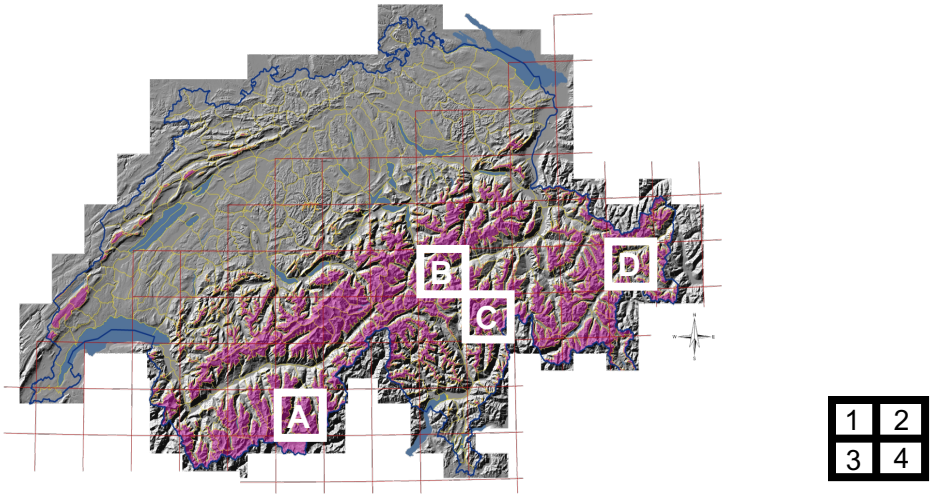


Fig. 1: Location of the study cells. Each cell (about 25x23km) consists of four subcells each with a size of about 12x12km. The particular regions are: A Valais, B Region in the Canton of Uri, C Region San Bernardino (Adula), D Swiss National Park, Engadine. These regions lie within the grid of the EU project IntraBioDiv (IBD, see text). The grid is indicated with thin lines. The inset shows the arrangement of the four subcells within each IBD cell.

Table 1: Restriction mix: Buffer 2, EcoRI and MseI are delivered by New England BioLabs (Bioconcept, Allschwil, CH), bovine serum albumin (BSA) by LaRoche (Basel, CH).

Components (stock concentration)	µl per sample
H_2O	12.35
Buffer 2 (10x)	2.00
BSA (1mg/ml)	0.20
EcoRI restriction enzyme (10U/µl)	0.20
MseI restriction enzyme (20U/µl)	0.25
DNA solution	5.00

Table 2: Ligation mix: T4 ligase buffer and T4 ligase are delivered by LaRoche (Basel, CH) and the adaptor mixes by Microsynth (Balgach, CH). For the sequence of the adaptors see text.

Components (stock concentration)	µl per sample
H_2O	12.79
T4 ligase buffer (10x)	4.00
EcoRI adaptor mix (10µM)	1.44
MseI adaptor mix (10µM)	1.44
T4 ligase (5Uµl)	0.20
Restriction product	20.00

Table 3: Mix for the preselective PCR. Buffer and $MgCl_2$ are delivered by Sigma (Buchs, CH), primers by Microsynth (Balgach, CH) and the AmpliTaq® DNA Polymerase by Applied Biosystems (Foster City, USA). See text for the sequence of the primers.

Components (stock concentration)	µl per sample
H_2O	14.90
PCR-Buffer (10x)	2.50
$MgCl_2$	1.50
dNTPs	2.00
Primer: EcoRI-presel (10µM)	0.50
Primer: MseI-presel (10µM)	0.50
AmpliTaq® DNA Polymerase (5U/µl)	0.10
Diluted ligation product (1:10)	3.00

Table 4: Cycles of the preselective PCR

Temperature (°C)	Duration	Replication
72	2 min	
94	30 sec	
56	30 sec	30 cycles
72	2 min	
72	10 min	

Table 5: Selective bases of the primers for the selective PCR, number of populations and the laboratory where the AFLP procedure has been performed.

Species	Abbreviation	Selective bases	Number of populations	Laboratory
Arabis alpina	Aal	AAT-CAC AGC-CAC ATC-CAC	13	Grenoble
Carex sempervirens	Cse	ACA-CAC ACA-CTG ATG-CAG	13	WSL
Dryas octopetala	Doc	AAC-CTG ACT-CTG ATG-CTG	14	Grenoble
Geum montanum	Gmo	ACA-CAC ACA-CTG ACC-CAT	16	WSL
Geum reptans	Gre	ACA-CAC ACA-CAT ACC-CAT	13	WSL
Gypsophila repens	Gyr	ACA-CT ACT-CA ATG-CT	11	WSL
Hypochoeris uniflora	Hun	ACA-CAC ACC-CAG AGG-CTG	7	Grenoble
Juncus trifidus	Jtr	ACA-CA ACT-CT ATG-CT	13	WSL
Loiseleuria procumbens	Lpr	AAT-CTG ACT-CTG ATG-CAC	14	Grenoble
Rhododendron ferrugineum	Rfe	AAT-CAC ATC-CAC ATG-CTG	16	Grenoble

Table 6: Mix for the selective PCR. Buffer, $MgCl_2$, the selective EcoRI primer and the Ampli TaqGold Polymerase are delivered by Applied Biosystems (Foster City, USA), the selective MseI primer by Microsynth (Balgach, CH) and the bovine serum albumine (BSA) by LaRoche (Basel, CH). The sequences of the selective primers are indicated in Table 5.

Components (stock concentration)	µl per sample
H_2O	11.60
Buffer (10x conc.)	2.50
$MgCl_2$	2.50
dNTPs	2.00
EcoRI-sel (10µM)	0.50
MseI-sel (10µM)	0.50
BSA (1mg/ml)	0.20
Ampli TaqGold Polymerase (5U/µl)	0.20
Diluted preselective PCR product	5.00

Table 7: Cycles of the selective PCR

Temperature (°C)	Duration	Replication
95	10 min	
94	30 sec	
65-56	1 min	13 cycles
(decrease of 0.7°C per cycle)		
72	1 min	
94	30 sec	
56	1 min	23 cycles
72	1 min	
72	10 min	

Results

The three primer combinations yielded a total of about 100 reliable markers per species (Table 8). The particular species had a very different proportion of polymorphic loci. For *Carex sempervirens*, *Dryas octopetala* and *Geum montanum*, the proportion of polymorphic loci lies at more than 90%. Other species had not much more than 50% polymorphic loci (*Arabis alpina*, *Hypochoeris uniflora*). The percentage of polymorphic loci in *Geum reptans, Gypsophila repens, Juncus trifidus, Loiseleuria procumbens* and *Rhododendron ferrugineum* was between 67 and 76% (Table 8).
For two samples of each species, extraction and AFLP procedure were performed twice on the same plate. The repeatability of the data was higher than 95%. In more than half of the samples, the pattern of the fragments was reproducible with 100%.
Average gene diversity according to Nei (1973) was calculated for each population of all species. The average gene diversity per species reached values between 0.03 for *A. alpina* and 0.11 for *Geum montanum* (Fig. 2).

I compared average gene diversities of the samples I collected in the subcells with average gene diversities obtained from the cell samples of IntraBioDiv. Twenty cell diversity values out of 32 were within the 95% confidence interval of the values from the individuals of the subcell samples. The other diversity values of the large grid were either above or below the upper or lower confidence interval boundaries of the subcell samples (Fig. 3).
In order to combine the results over all species sampled, I calculated mean average gene diversity over all species per IntraBioDiv grid cell (Fig. 4) and compared it with that of the subcell samples. In three cells, cell diversities were higher than the 95% confidence interval of the subcell samples, while one value was lower. The highest mean average gene diversity of the cell samples was reached in the cell including the Swiss National Park, where the largest difference between diversities of the two spatial scales could be detected.

For the down-scaling approach, I calculated mean average gene diversity only taking into account mean diversity within the IntraBioDiv grid cells. I calculated the sum of deviances from mean average gene diversity per species for each subcell (see Methods). Average gene diversity per subcell was not distributed with a clear pattern. Three of the subcells with the largest negative deviances belonged to the grid cell in the Swiss National Park. In the grid cell Adula, three of the four diversity values were higher than in other cells (Fig. 5).

Most of the species were not genetically differentiated among the study sites. The highest partition of total genetic variation was found within populations. *Dryas octopetala, Geum montanum, Gypsophila repens, H. uniflora, J. trifidus* and *L. procumbens* were not subdivided into two or more genetically distinct groups by the software *Structure* (Pritchard *et al.*, 2000). Those species had F_{ST} values ranging from 0.00 to 0.17 (Table 9). *Loiseleuria procumbens* showed no variation among populations. Four species were genetically more differentiated and can be assigned to two groups (*C. sempervirens, Geum reptans, R. ferrugineum* and *A. alpina*). *Carex sempervirens*, *Geum reptans* and *R. ferrugineum* had F_{ST} values between 0.29 and 0.39 (Table 9). These values include variation among the two groups as well as variation among populations within groups. As an exception, *A. alpina* had a very high F_{ST} value of 0.69 with more variation among populations than within populations (Table 9).

The analysis of the genetic structure of *Geum reptans* with *Structure* software version 2.0 (Pritchard *et al.*, 2000) yielded a subdivision of the populations into two genetically separated groups. The geographic distribution of these two groups showed a western and an eastern group (Fig. 6). All populations of the IntraBioDiv cell Valais and one population of the grid cell Uri belonged to the western group, whereas the other populations were assigned to the eastern group. I combined my data set of *Geum reptans* with that of Thiel-Egenter *et al.* (in prep.), which included populations of the whole range of the Alps and the Carpathians. Analyzing the whole dataset with *Structure* software (Pritchard *et al.*, 2000) showed that the subcell samples fit well into the population structure from the entire Alps (Fig. 7). The most western population of my samples in the Valais was placed into a more western group of the IntraBioDiv samples, and the

populations in the Swiss National Park were partly assigned to a group located east of Switzerland.

The inference of the population structure of *C. sempervirens* in my data set revealed two groups (Fig. 8). One group consisted of three populations located in the grid cell of the Swiss National Park. The other ten populations, located in the other IntraBioDiv grid cells (Uri, Adula, Valais) and in one subcell of the IntraBioDiv grid cell Swiss National Park, respectively, belonged to a separate group.

Rhododendron ferrugineum also showed a pattern of two genetically separated groups (Fig. 9). The pattern was similar to that of *C. sempervirens*, as three populations located in the grid cell Swiss National Park belonged to a genetically separated group. Again, one population from the Swiss National Park was different from the three mentioned ones and was assigned to the second group which included all other populations in the grid cells Valais, Uri and Adula. In the grid cell Swiss National Park, the populations of *C. sempervirens* and *R. ferrugineum* which were not assigned to the eastern group of their species were located in subcell 1 for *Carex* and 3 for *Rhododendron*, respectively.

The inference of population structure of *A. alpina* revealed not a clear geographical distribution with rather a North-South than an East-West distribution (Fig. 10).

Table 8: Number of AFLP markers per species and percentage of polymorphic loci

Species	Number of markers	% polymorphic loci
Arabis alpina	93	58.1
Carex sempervirens	120	95.8
Dryas octopetala	105	90.5
Geum montanum	89	94.4
Geum reptans	88	67.0
Gypsophila repens	109	72.5
Hypochoeris uniflora	97	52.6
Juncus trifidus	113	72.6
Loiseleuria procumbens	106	76.4
Rhododendron ferrugineum	110	72.7

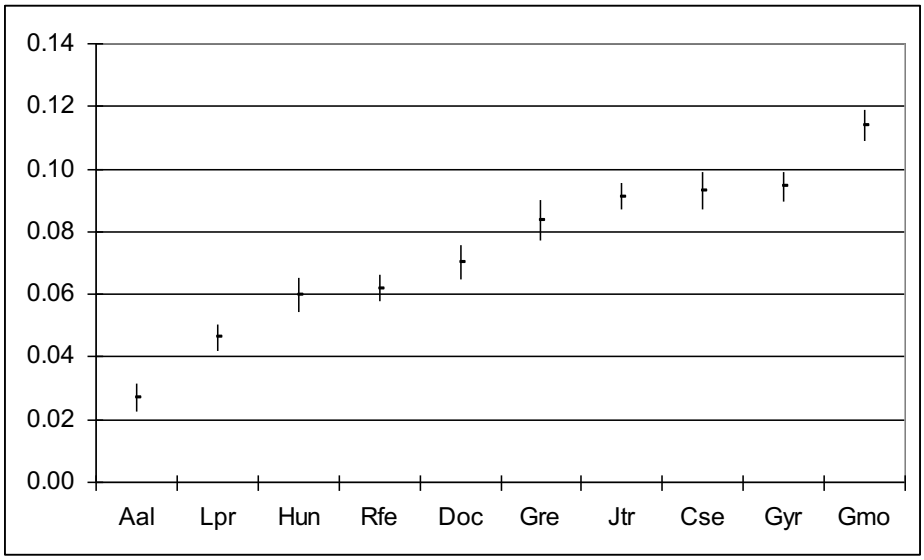


Fig. 2: Mean value of Nei's average gene diversity per species. Vertical bars indicate the standard error. Aal = *Arabis alpina*, Cse = *Carex sempervirens*, Doc = *Dryas octopetala*, Gmo = *Geum montanum*, Gre = *Geum reptans*, Gyr = *Gypsophila repens*, Hun = *Hypochoeris uniflora*, Jtr = *Juncus trifidus*, Lpr = Loiseleuria procumbens, Rfe = *Rhododendron ferrugineum*.

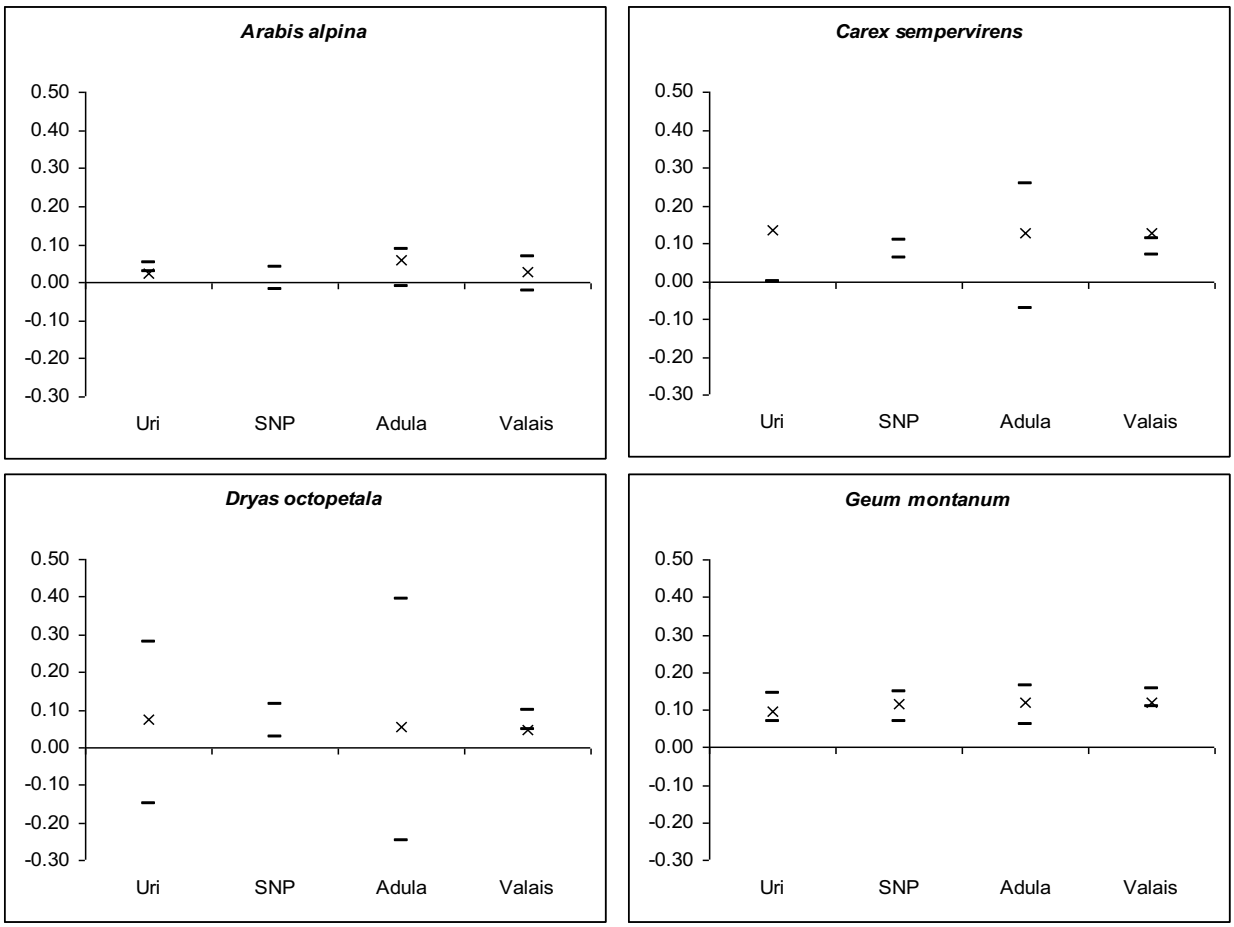
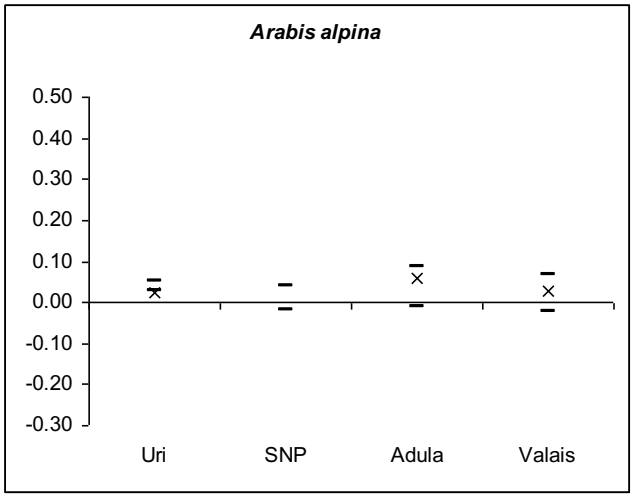


Fig. 3: Continued and described on the next page.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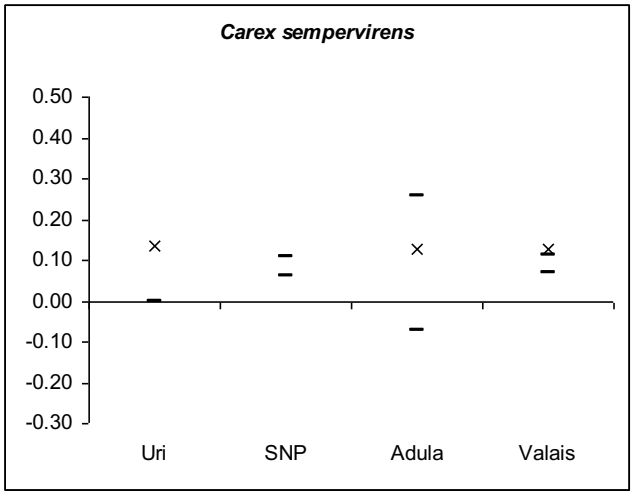
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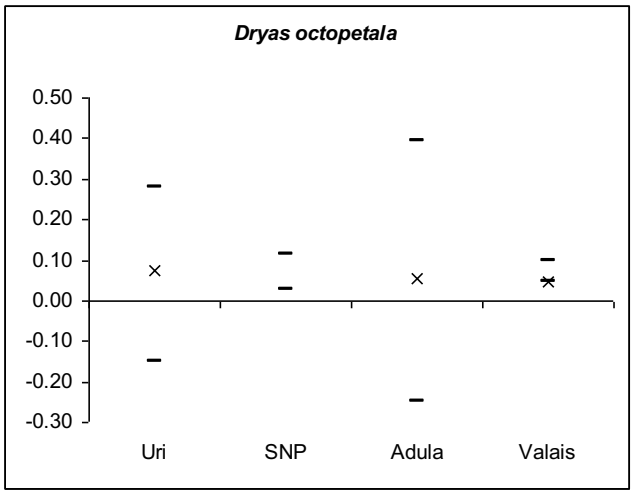
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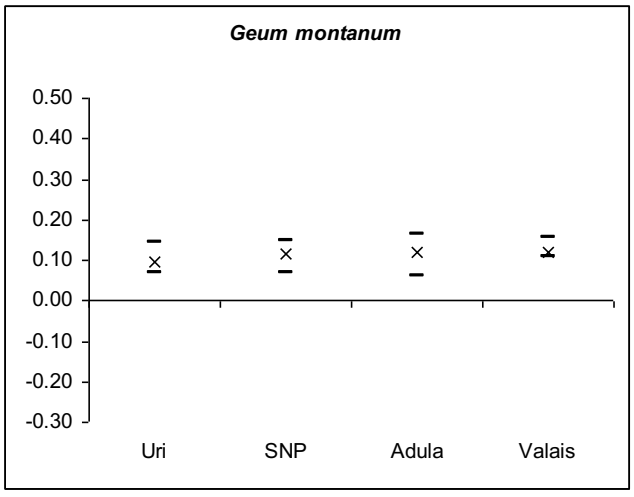
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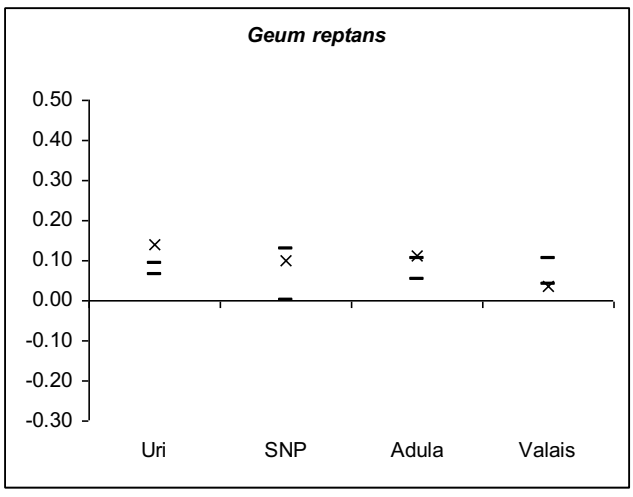
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0.10
0.00
-0.10
-0.20
-0.30
Uri
SNP
Adula
Valais

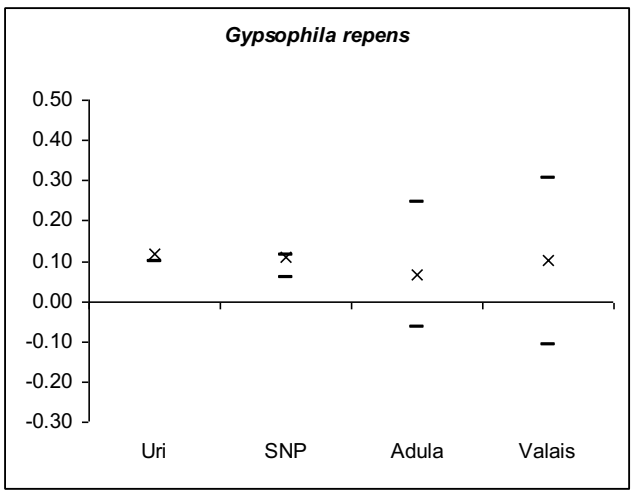
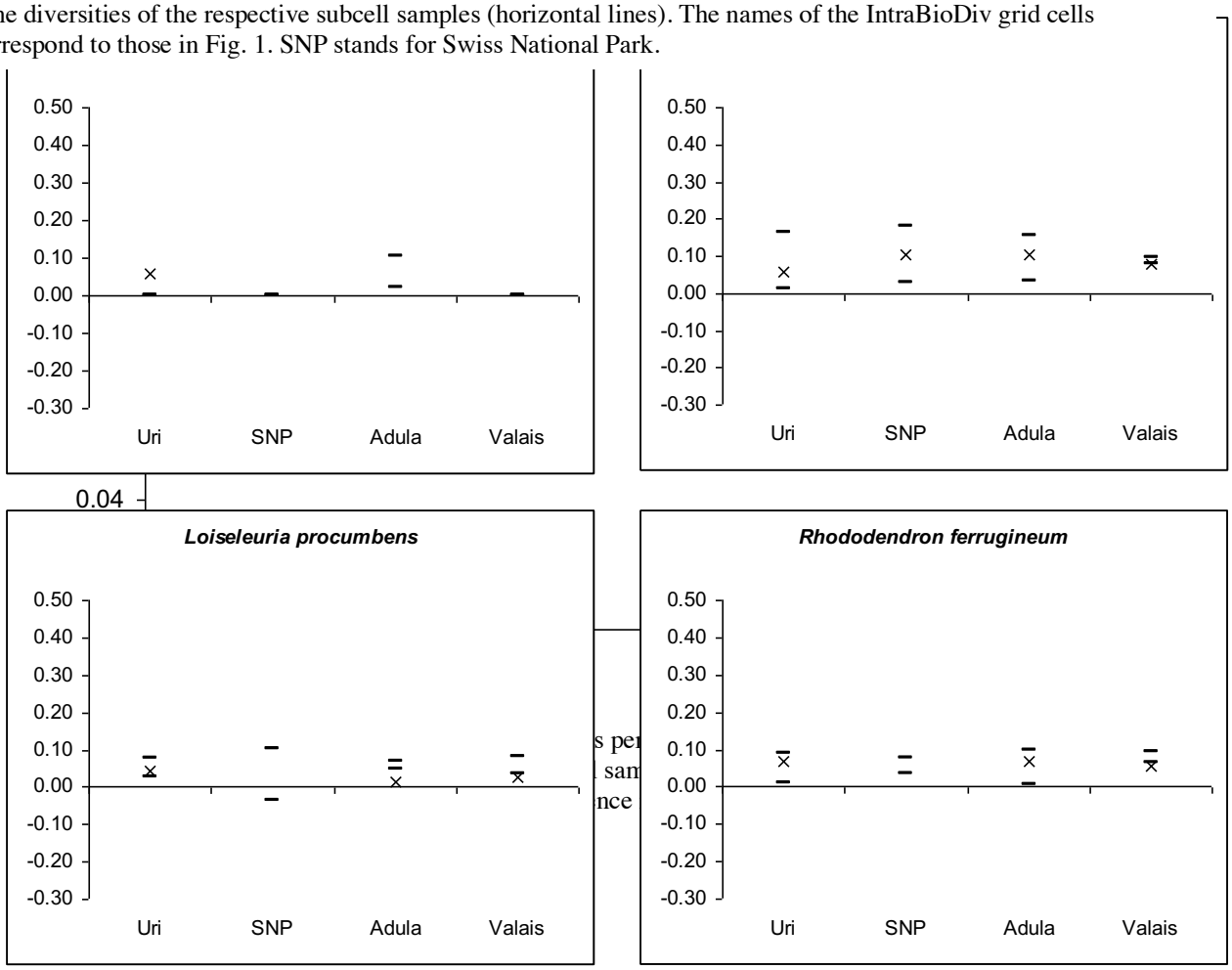

Gypsophila repens
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
Uri
SNP
Adula
Valais

Fig. 3: Average gene diversity per IntraBioDiv grid cell for each species (crosses) and 95% confidence interval of gene diversities of the respective subcell samples (horizontal lines). The names of the IntraBioDiv grid cells correspond to those in Fig. 1. SNP stands for Swiss National Park.



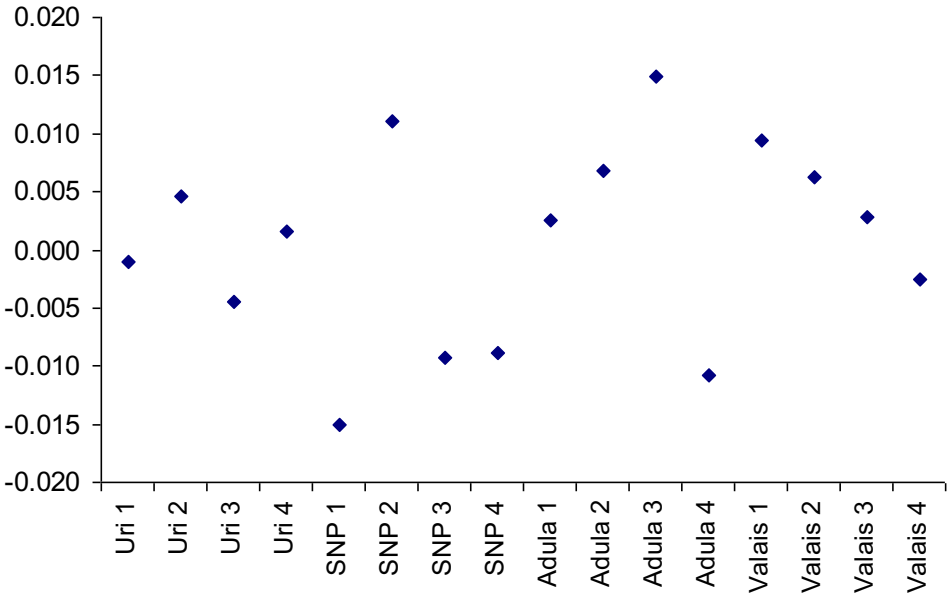


Fig. 5: Weighted sum of deviances from the mean average gene diversity per species calculated for each subcell. For further explanation of the calculation of these deviances see text (material and methods).

Table 9: Source of variation and F_{ST} values of the study species. The number of groups refers to the population structuring inferred with *Structure* software (Pritchard *et al*., 2000).

Species	Number of groups	Source of variation [%]			F_{ST}	p-value
		Among groups	Among populations (within groups)	Within populations		
Arabis alpina	2	16.21	52.84	30.95	0.69	0.00
Carex sempervirens	2	30.40	8.87	60.73	0.39	0.00
Dryas octopetala	1		3.23	96.77	0.03	0.02
Geum montanum	1		12.58	87.42	0.13	0.00
Geum reptans	2	13.17	16.65	70.19	0.30	0.00
Gypsophila repens	1		17.30	82.70	0.17	0.00
Hypochoeris uniflora	1		15.39	84.61	0.15	0.00
Juncus trifidus	1		6.39	93.61	0.06	0.00
Loiseleuria procumbens	1		0.00	100.00	0.00	0.12
Rhododendron ferrugineum	2	14.30	14.35	71.35	0.29	0.00

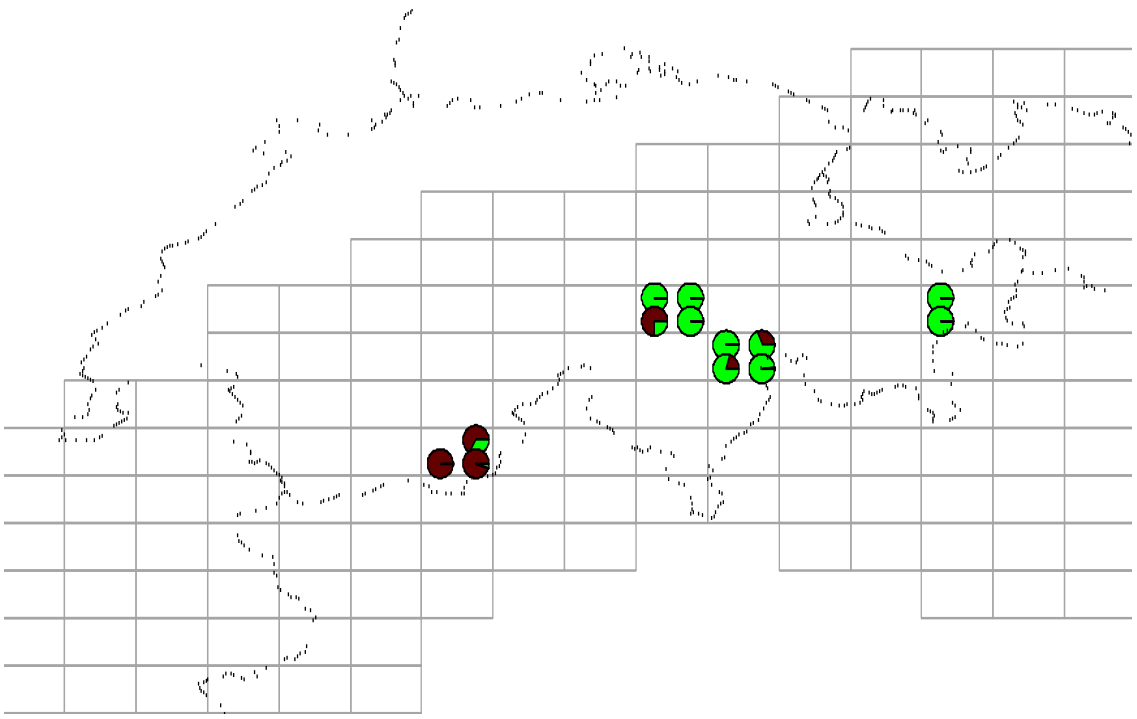


Fig. 6: Population structure of *Geum reptans* inferred with *Structure* software (Pritchard e
Grey lines indicate the grid of the EU project IntraBioDiv.

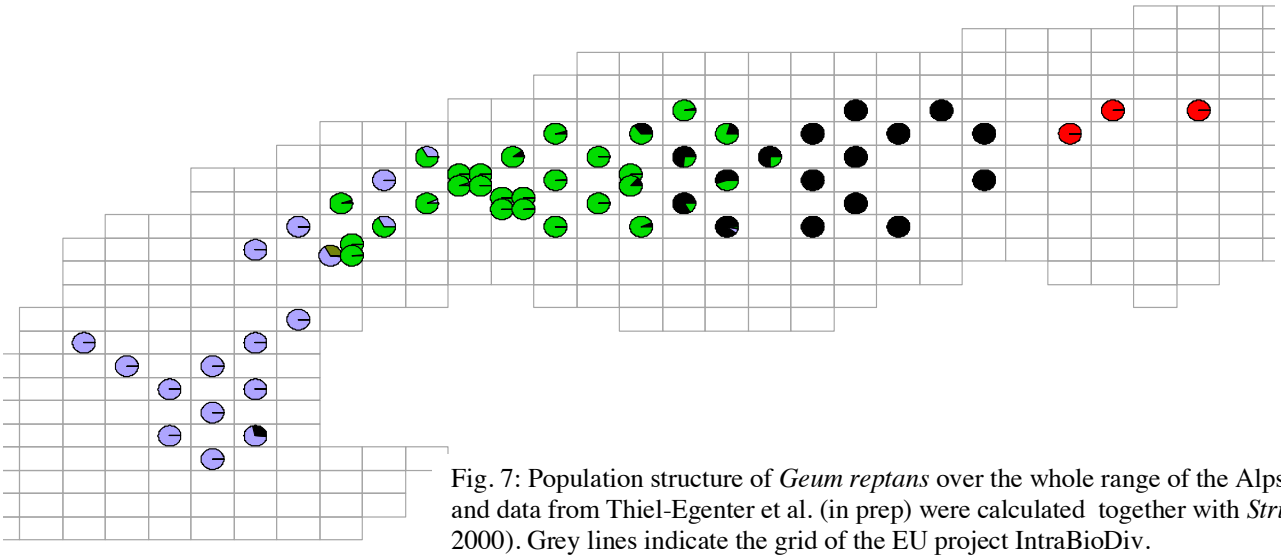


Fig. 7: Population structure of *Geum reptans* over the whole range of the Alps. Data from the
and data from Thiel-Egenter et al. (in prep) were calculated together with *Structure* software
2000). Grey lines indicate the grid of the EU project IntraBioDiv.

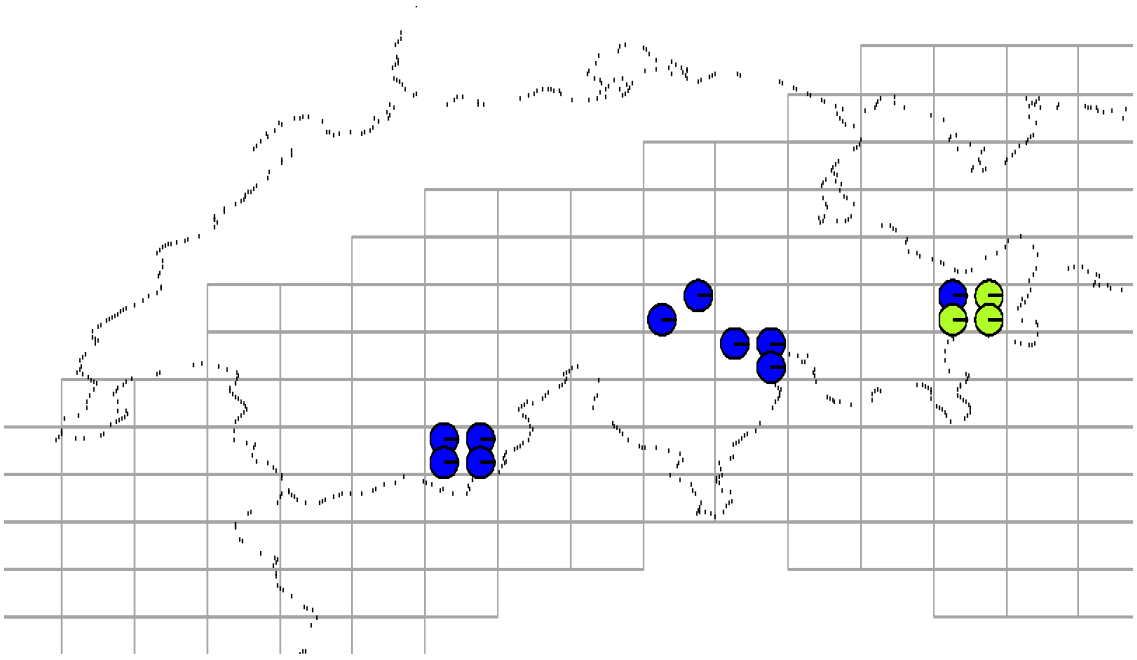


Fig. 8: Population structure of *Carex sempervirens* inferred with *Structure* software (Pritchard *et al*., 2000).
Grey lines indicate the grid of the EU project IntraBioDiv.

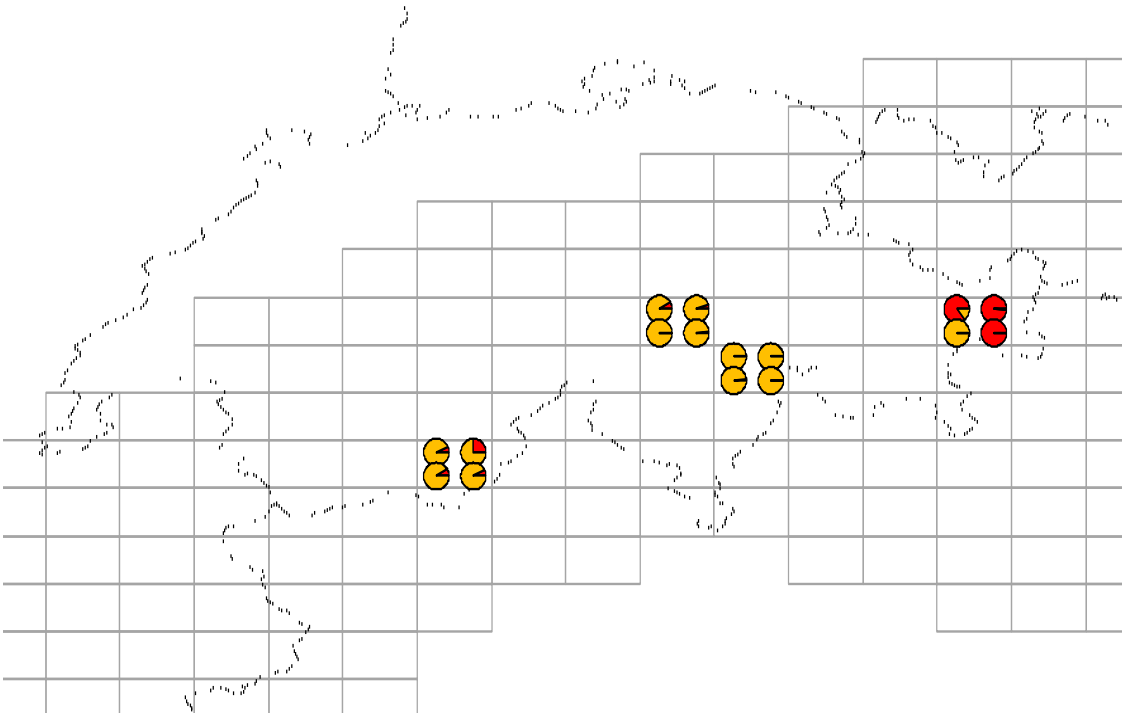


Fig. 9: Population structure of *Rhododendron ferrugineum* inferred with *Structure* software (Pritchard *et al*., 2000). Grey lines indicate the grid of the EU project IntraBioDiv.

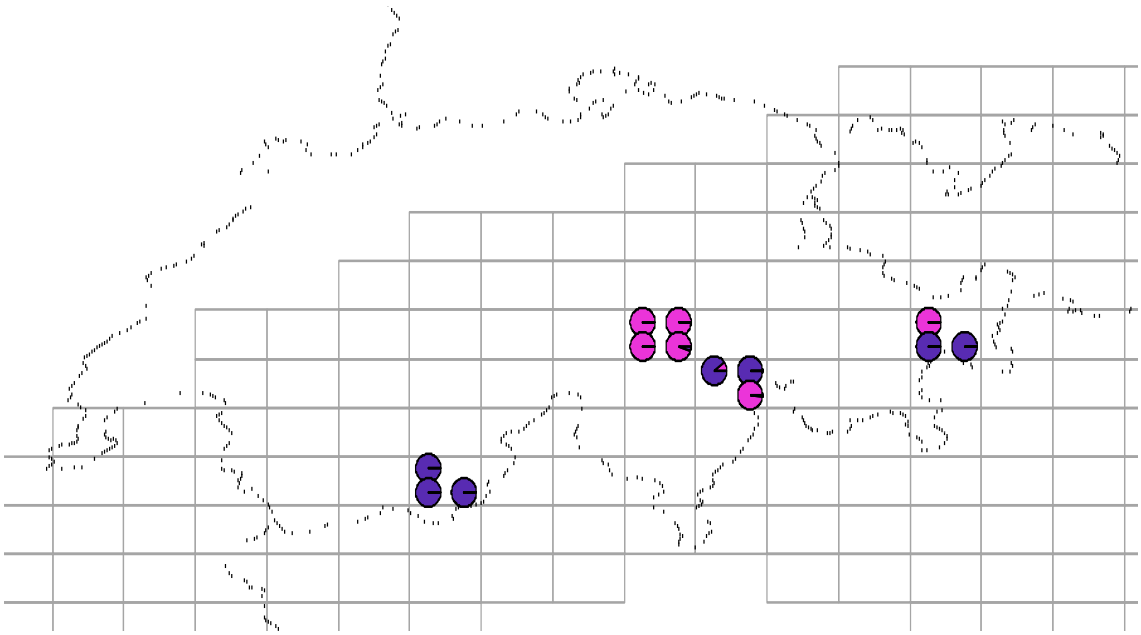


Fig. 10: Population structure of *Arabis alpina* inferred with Structure software (Pritchard *et al*., 2000). Grey lines indicate the grid of the EU project IntraBioDiv.

Discussion

Down-scaling / diversity per species

The down-scaling analysis of genetic diversity generally yielded a good correlation on the two spatial scales. In half of the species, the values of genetic diversity of the subcells corresponded well to those of the cell samples. The other species showed either slightly higher or lower values of genetic diversity on the small than on the large scale.
Previous studies of genetic diversity are mostly performed by irregularly sampling across a particular landscape. The sampled populations should at best cover the whole study area (Gaudeul *et al*., 2000; Stehlik *et al*., 2001). However, an alternative possibility to investigate genetic diversity of plant species in a given area is to lay a grid over the whole area and collect populations of the study organisms according to the defined grid. This approach has been chosen by the EU project IntraBioDiv, which investigates genetic diversity of 27 alpine plant species over the whole range of the Alps and the Carpathians. The dimension of the grid cells used in the study to sample individuals appears as quite large (25x23km). However, for the analysis of genetic diversity, taking into account the whole range of the Alps, this likely represents an appropriate resolution due to the large number of populations included in the analysis. The down-scaling to four times smaller grid cells of about 12x12 km has been done for all ten study species. I strictly followed the sampling design of the EU project IntraBioDiv to allow for a comparison with the results of IntraBioDiv.
The comparison of genetic diversity of the cell samples (large scale) with that obtained from the subcell samples (small scale) led to different results depending on the species. In some species, the mean average gene diversities of the cell samples corresponded very well to that of the subcell samples. Namely *Arabis alpina*, *Dryas octopetala*, *Geum montanum*, *Gypsophila repens* and *Juncus trifidus* showed a good correlation between the genetic diversity of the two different scales (Fig. 3). On the other hand, the diversities of *Carex sempervirens*, *Geum reptans*, *Hypochoeris uniflora*, *Loiseleuria procumbens* and

Rhododendron ferrugineum did not fit into the 95% confidence interval of genetic diversity of the subcell samples. In *Geum reptans*, for example, only one value of mean average gene diversity of the cell samples lay within the 95% confidence interval of mean average gene diversity of the subcell samples (Fig. 3). This indicates that we should be cautious when comparing diversity measures of two different spatial scales. However, a study of *Geum reptans* in the Swiss Alps shows that the amount of diversity within populations is irrespective of population origin (Pluess, Stöcklin, 2004). In contrast, Thiel-Egenter *et al*. (in prep.) analyzed genetic diversity from over the entire Alps and found an unequal distribution, indicating that genetic diversity is influenced by factors that act on large distances. This finding is also supported by Gugerli *et al*. (2001) for *Picea abies* and by Schönswetter *et al*. (2004) for *Ranunculus glacialis*, which found an unequal distribution of biodiversity on a large scale. In my study I found for *Geum reptans* diversity values of the large scale that are higher, lower or similar to those of the small scale (Fig. 3). Therefore, I would expect an equal distribution of diversity if the study was expanded to more than the four grid cells studied here.
Since the comparison of genetic diversity on two different scales leads to inconsistent results for single species, a more integrative approach, i.e. calculating genetic diversity by averaging over all study species, likely provides a more general perspective.

Down-scaling / diversity per cell and subcell

When taking all ten study species into account, the mean average gene diversity of three cell samples were higher and one was lower than the values obtained from the subcell samples (Fig. 4). The largest difference was found in the grid cell of the Swiss National Park. This might be due to only four species contributing to the diversity of the cell samples which are weighted higher. The species all have similar but high diversities in the grid cell Swiss National Park (Fig. 4).
However, the above mentioned differences between the diversities of the cell samples and the subcell samples are influenced by the diversities in the particular subcells. Genetic diversity of populations is not expected to be equally distributed. One of the

main factors driving genetic diversity of populations is population size (Fischer, Matthies, 1998; Gaudeul *et al*., 2000). Since I collected only three species per study cell without taking into account the effective population size, this could possibly explain part of the differences in diversity measures. The analysis of diversity in the single subcells did not reveal a clear pattern (Fig. 5). The grid cell Swiss National Park seemed to exhibit a low diversity. Three of the subcells with the lowest diversity belonged to this grid cell (Fig. 5). The subcell with the highest diversity was located in the grid cell Adula, but other subcells of this grid cell showed a lower diversity. Likewise, this was the case in the other grid cells, with high as well as low diversity values in the particular grid cells. Therefore, I conclude that the diversity values of the four grid cells are not falsified by some extreme high or low diversity values of single subcells. It would be interesting to perform a down-scaling approach in more grid cells. Then, we could evaluate whether the different diversity values of the cell samples and the subcell samples in the grid cell Swiss National Park are outliers or whether there are some more grid cells which exhibit a different amount of genetic diversity on the two spatial scales.

Diversity per species

As discussed above, the distribution of genetic diversity on two spatial scales was not consistent over all study species. Differences were also expected and found in the absolute amount of genetic diversity per species. For example, *A. alpina* showed the smallest genetic diversity of all species studied (Fig. 2). This is consistent with Koch *et al*. (2006) who showed a very low diversity of nuclear as well as chloroplast markers of *A. alpina* in the Alps. The diversity value of my analysis from *D. octopetala* is also supported. Skrede *et al*. (2006) found a similar amount of genetic diversity in a population in the French Alps.

Even if some diversity measures found were supported by other studies, the differences between the particular species were obvious. The diversity values of the ten study species ranged from 0.03 for *A. alpina* to 0.11 for *Geum reptans*. These differences are likely to occur because each species has a particular combination of life history traits which are

known to have an influence on the genetic diversity of the single species (Hamrick, Godt, 1996; Nybom, Bartish, 2000).

The results of my analysis indicate a correspondence between genetic diversity and pollination strategy. This is in congruence with the assumption that genetic diversity in wind-pollinated species is higher because the pollen is transported over larger distances compared to insect-pollinated species, thus increasing genetic diversity within species (Loveless, Hamrick, 1984). In my study species, the values of genetic diversity in the wind-pollinated plants *C. sempervirens* and *J. trifidus* were in the upper part of the distribution of genetic diversity regarding all ten study species.

The distribution of genetic diversity among my ten study species could also be influenced by the substrate. Calcareous and siliceous bedrocks do not occur regularly over the Alps. Therefore, species that depend on a particular substrate do not necessarily find a suitable habitat in their neighborhood and possibly need to disperse over greater distances. It is known that in Alpine habitats the species richness is dependent on the substrate (Wohlgemuth, 2002). A calcareous substrate is favoured by more species than a siliceous substrate (Wohlgemuth, 2002). Given that processes influencing species richness do also affect genetic diversity, it is reasonable to assume that the substrate has an influence on the genetic diversity of species. However, analyzing my ten study species revealed no coincidence between substrate demand and genetic diversity. For example, the calciphilous species *A. alpina* has the lowest diversity value of the ten study species, whereas *Gypsophila repens*, which also grows exclusively on calcicolous substrate, was among the most genetically divers species. This indicates that the amount of genetic diversity in the ten study species is not affected by the particular substrate demand.

Even if some diversity measures found were supported by other studies, it is noteworthy that the five species with the lowest diversity values (*A. alpina*, *D. octopetala*, *H. uniflora*, *L. procumbens*, *R. ferrugineum*) were all handled by the laboratory in Grenoble to perform the AFLP procedure. Even though the two laboratories (WSL, Grenoble) used the same AFLP protocols, some differences seem possible. This concerns the use of PCR machines, centrifuges or other equipment which may affect the laboratory work, particularly when screening random markers. However, Jones *et al.* (1997) tested the

reproducibility of AFLPs across several European laboratories and found that only one band out of 64 was not reproducible. A further factor that could influence AFLP procedures is the amount of DNA used for restrictions or PCRs. The band pattern may be different if DNA is excessively diluted (Vos *et al*., 1995). Since I did all extractions in the laboratory at WSL, the dilution of DNA was constant over all study species. It is important to mention that I analyzed the band patterns for all ten study species, thus avoiding a bias in the data owing to electropherogram interpretation. However, since there was an apparent difference between the amounts of genetic diversity of the particular species, it might be reasonable not to interpret the absolute amount of genetic diversity of the species. This can be achieved by calculating mean average gene diversity taking into account all study species or by regarding the distribution within species rather than between species. If more than one laboratory is included in the AFLP process it could be helpful for future investigations to do the laboratory work for some individuals in both labs. Then the differences would be obvious and the effect of the particular lab could be estimated.

Genetic differentiation

It is not only diversity that is a relevant component of the genetic structure of a species, but also genetic differentiation. Genetic differentiation was shown to be different between three spatial scales (Rivera-Ocasio *et al*., 2006). These authors found an increase in among-population differentiation by increasing spatial scales. They investigated genetic differentiation on scales of less than 10 km to more than 1000 km. Even though it is partly a larger scale compared to the current study, it indicates a possible constraint of the genetic differentiation of populations obtained on different spatial scales. An incongruence between different spatial scales was also found in the genetic analysis of *R. ferrugineum* (Wolf *et al*., 2004). These authors found no correlation between genetic and spatial distance on a large scale, but along a local transect, which suggests that gene flow and genetic drift may be in equilibrium below the scale of 2 km. A study on *D. octopetala* by Skrede *et al*. (2006) shows a high amount of genetic diversity within

populations (ca. 80%) and a low diversity among populations on a global scale. Therefore, it is not surprising that on a smaller scale (as used in the current study) the diversity among populations is smaller due to a higher likelihood of gene flow between the populations as a result of the smaller distances.

The partition into smaller grid cells gives a better resolution of genetic structure and is perhaps not representative for a larger scale. Inferring population structure of *R. ferrugineum* of my data with *Structure* software (Pritchard *et al*., 2000) yielded two genetically separated groups. One group consisted of three populations in the grid cell of the Swiss National Park and the second group of all other populations (Fig. 9). It is notable that the population of *R. ferrugineum* located in the Val Trupchun did not group with the other populations in the grid cell Swiss National Park, which were sampled near the Ofenpass, in the upper part of the Val Sampuoir and near Alp Laschadura. Considering this geographical situation, it is not surprising that the population in the Val Trupchun was grouped differently. The mountainous ridge that separates the population in the Val Trupchun from the other populations in the grid cell Swiss National Park possibly acts as a barrier for gene flow, whereas the other three populations of this cell may rather be connected via gene flow. The genetic separation of populations in the grid cell Swiss National Park is in congruence with Manel *et al*. (2005) who investigated genetic discontinuities of *R. ferrugineum* in the Alps and found three main populations with transition zones between them. The grid cell Swiss National Park lies in the transition zone between the two main populations of the central Alps and the eastern Alps.

A similar pattern of genetic differentiation was found for *C. sempervirens*. As in *R. ferrugineum*, there are three populations in the grid cell Swiss National Park which make up a genetically separated group (Fig. 8). The difference to *R. ferrugineum* is the distribution within the grid cell. In *C. sempervirens*, the population growing on the mountain system of the lakes of Macun is separated from the other populations of this cell. The Swiss National Park consists mainly of dolomite and is therefore an area for basophilic plant species. Only in the region of Macun there are crystalline rocks, which provide a substrate for acidophilic plant species. *Carex sempervirens* is a plant species

that prefers calcareous substrates but sometimes it occurs on siliceous bedrock. One can imagine that the preference of a particular substrate is written down in the genetic information of the species. This could explain that the population from the siliceous mountain system was genetically separated from the calcareous ones.

The analysis of genetic structure of *Geum reptans* revealed a partition into two genetic separated groups (Fig. 6). The border between these groups was not located in the east of Switzerland as in *C. sempervirens* and *R. ferrugineum*, but further west. The populations in the grid cell Valais made up one group. The population in the southwestern subcell in the grid cell Uri was also assigned to the western group, even if the geographic distance between this population and those in the Valais is quite large. It would be interesting to take into account more grid cells between the Valais and Uri to investigate the genetic structure of populations distributed over the whole range of the Swiss Alps.
I combined my data set of *Geum reptans* with that of Thiel-Egenter *et al*. (in prep.) which included populations of the whole range of the Alps (Fig. 7). Nearly all populations of my study were assigned to a single group. Only the most western population of my samples, namely the one in the region of Zermatt, was assigned to a group located more west of Switzerland. It is notable, but not necessarily surprising, that the analysis of only my samples did not reveal the same grouping as the analysis together with the data set of the entire Alps. Just analyzing my samples indeed revealed two groups, but the genetic distance between these two groups was possibly too small to be detected in a larger data set.
Overall, the genetic structure of these three species (*R. ferrugineum*, *C. sempervirens* and *Geum reptans*) showed a differentiation of the populations into a western and an eastern group. This is in congruence with Schönswetter *et al*. (2005) who found a biogeographic separation in the east of Switzerland. *Carex sempervirens* and *R. ferrugineum* fitted very well to this line with the separated populations in the eastern part of Switzerland, whereas the line obtained for *Geum reptans* was shifted towards the western part of Switzerland. However, the other study species did not support the position of this line, either because they are not divided into two groups or because the populations were not divided into an eastern and a western group. The latter case was found in *A. alpina* which did not group

with a similar pattern compared with the above mentioned species by analyzing with *Structure* software (Pritchard *et al*., 2000). The populations of *A. alpina* were assigned to two genetically separated groups, but this grouping revealed rather a North-South than an East-West distribution (Fig. 10). It is difficult to interpret the distribution of these two genetically separated groups. Schönswetter *et al*. (2005) indicate that potential glacial refugia of alpine plants growing on calcareous substrate can be found in the northern and the southern border of the Alps. Therefore, the distribution of genetically separated groups as found in the current study could result from the distribution in respective marginal refugia during the last ice age. One can imagine that the species survived in those refugia and began to immigrate from both sides into the Alps as the glaciers melted. The area in the center of the Alps could represent a contact zone of the formerly separated populations. However, the number of populations and the study area should be expanded for such an investigation.

In summary, alpine plant species show different amounts of genetic diversity, and the respective values may depend on the spatial scale studied. The down-scaling from a large grid to a four times smaller one did not reveal a clear distribution of genetic diversity. Diversity measures coincided well between the two spatial scales in some species, but not in others. Therefore, it would be important to include an even higher number of species when investigating down-scaling in a larger area or by taking into account more than the four grid cells studied here. With more species, the influence of single species can be reduced.

Even though I detected only a weak correlation between genetic diversity and pollination strategy, it seems that the two parameters are correlated. More wind-pollinated species should be included to verify this correlation.

Even though the AFLP procedure is well known for its reproducibility, there are some constraints when not the whole procedure is done in one lab. However, this should only affect the absolute amount of genetic diversity within a species, which may be accounted for when comparing different species.

Differentiation is a relevant component of genetic structure of a species. In four species, the study populations could be assigned to two genetically separated groups with a pattern of an East-West partition in three of them. This is in congruence with recent studies that detected biogeographic lines in the eastern part of Switzerland.

A majority of the study sites in the current study were located in areas of potential new national parks or the existing Swiss National Park. Therefore I will conclude with some indications of my results for conservation matters. Since genetic diversity seems to be correlated with the pollination strategy, it would be important for the establishment of protection areas to take into account areas that cover a large number of species which may exhibit different amounts of genetic diversity. Furthermore, diversity was not equally distributed over the whole study area. This may suggest that areas for protection of genetic diversity should include locations distributed over a large region. Thus, the strategy of Pro Natura to establish a new national park in Switzerland would be a good possibility to expand the already existing protection areas and therefore to maintain a larger amount of genetic diversity and differentiation of alpine plant species.

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