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Gradual decline in genetic diversity in Swiss stone pine populations (*Pinus cembra*) across Switzerland suggests postglacial re-colonization into the Alps from a common eastern glacial refugium

Felix Gugerli · Martin Rüegg · Giovanni G. Vendramin

Abstract Molecular genetic markers may reveal informative patterns of population processes such as historical migration, which may substantiate inference on postglacial re-colonization inferred, e.g., from fossil records. Palynological records of Swiss stone pine (*Pinus cembra*) suggest that the species has re-colonized the central Alps from a southeastern Alpine refugium after the last glacial maximum. Such a migration pathway likely resulted in a gradual decrease in genetic diversity with increasing distance to the glacial refugium, owing to founder events at the leading range edge. The present distribution of *P. cembra* in Switzerland consists of two rather distinct ranges, namely the inner-alpine parts of the Grisons and Valais, respectively, and additional disjunct occurrences in the northern and southern periphery of the Alps as well as between the two main ranges. We screened chloroplast microsatellite loci on 39 Swiss *P. cembra* populations and show that the genetic structure detected was congruent with a common ancestry from a single glacial refugium, likely located at the (south-)eastern periphery of the Alps. In contrast, our data rejected the alternative hypothesis of a distinct genetic separation of the two main ranges of Swiss stone pine in Switzerland. We further show that low genetic diversity within and high differentiation among peripheral populations in the northern Alps as well as the genetic differentiation between core and peripheral populations reflect genetic drift as a consequence of colonization history and limited gene flow by pollen and seed.

Zusammenfassung Molekulargenetische Marker enthalten wertvolle Information über Populationsprozesse wie historische Wanderungen, wodurch Annahmen zur postglazialen Wiederbesiedelung, beispielsweise abgeleitet von Fossilfunden, unterstützt werden können. Palynologische Funde von Arve (*Pinus cembra*) lassen vermuten, dass diese Art nach dem letzten glazialen Maximum von einem Refugium in den südöstlichen Alpen wieder in die zentralen Alpen eingewandert ist. Ein solcher Rückwanderungsweg dürfte aufgrund von Gründereffekten an der Wanderungsfront eine graduelle Verringerung der genetischen Variation mit zunehmender Distanz zum Glazialrefugium bewirkt haben. Die heutige Verbreitung von *P. cembra* in der Schweiz weist zwei deutlich getrennte Gebiete auf, nämlich inneralpine Bereiche der Kantone Graubünden und Wallis, mit jeweils isolierten Vorkommen in den nördlichen und südlichen Randalpen. Wir untersuchten Chloroplasten-Mikrosatelliten in 39 Schweizer Populationen von *P. cembra* und zeigen, dass die gefundene genetische Struktur übereinstimmt mit der Annahme einer gemeinsamen Abstammung aus einem einzigen Glazialrefugium, welches vermutlich am

F. Gugerli (✉) · M. Rüegg
WSL Swiss Federal Institute for Forest,
Snow and Landscape Research,
Zürcherstrasse 111, 8903 Birmensdorf, Switzerland
e-mail: gugerli@wsl.ch

Present Address:
M. Rüegg
Schmerikonerstrasse 5, 8733 Eschenbach, Switzerland

G. G. Vendramin
Istituto di Genetica Vegetale, CNR, Via Madonna del Piano 10,
50019 Sesto Fiorentino, Florence, Italy

(süd-)östlichen Rand der Alpen lag. Im Gegensatz dazu widerlegen unsere Daten die alternative Hypothese einer deutlichen genetischen Trennung der zwei Hauptvorkommen der Arve in der Schweiz. Im Weiteren zeigen unsere Resultate eine geringe genetische Variation innerhalb und einen hohen Differenzierungsgrad zwischen nördlichen Randalpenvorkommen, sowie eine genetische Differenzierung zwischen zentralen und peripheren Populationen. Dies weist auf genetische Drift hin, welche die Besiedlungsgeschichte und einen beschränkten Genfluss durch Pollen und Samen widerspiegelt.

Introduction

Patterns of species and gene occurrences and diversities have been strongly shaped by climate oscillations during the Pleistocene. The repeated advance and retreat of glaciers and the area covered by the ice shields have determined areas of species survival during cold periods and also possible routes for re-colonization (Hewitt 2000; Brewer et al. 2002). These historical imprints have long been traced using floristic and biogeographical inference (Tribsch 2004). Lately, molecular techniques have enabled us to unravel these range shifts based on the extant pattern of genetic diversity within species (phylogeography; Avise 2009). For this purpose, organellar DNA markers, i.e. gene loci from the mitochondrial (mt) or the chloroplast (cp) genomes, have proved particularly informative (Petit and Vendramin 2007). Both cytoplasmic genomes are haploid, in general uniparentally inherited, and transmitted without recombination. Accordingly, haplotypes represent parental lineages that can be readily identified, their pedigree being determined mainly by rare mutations and their spread. Therefore, organellar DNA markers reflect the long-term genealogy of a species in a spatial context. Plant mtDNA is maternally inherited in practically all species, and the same holds for cpDNA in most angiosperms (Petit and Vendramin 2007). Most gymnosperms, however, show paternal inheritance of cpDNA (Petit and Vendramin 2007). Accordingly, maternally transmitted organellar genomes are dispersed by seed only, while paternally inherited cpDNA of gymnosperms is first dispersed by pollen and subsequently by seed as part of the paternal contribution to the embryo. These properties need to be considered when interpreting respective phylogeographic structures.

Countless studies have been performed in the past two decades to elucidate the glacial survival of plant species (Gitzendanner and Soltis 2000; Hewitt 2004; Soltis et al. 2006). In Europe, forest trees have received particular attention (Liepelt et al. 2002; Petit et al. 2002, 2003; Cheddadi et al. 2006; Magri et al. 2006; Tollefsrud et al. 2008), as they have been substantially affected by the mostly east–west oriented mountain ranges of Europe acting as barriers to the northward migration at the end of the Pleistocene ice age (Hewitt 1996).

As a consequence of the shift of a species' range after glaciation, populations at the margin of diffuse expansion or even at the leading-edge tend to be genetically less diverse than populations in or close to the refugial areas (Tollefsrud et al. 2008; but see Comps et al. 2001; Petit et al. 2004). This trend of declining diversity is the result of genetic drift, as, for example, evident in Norway spruce *Picea abies* (L.) Karst. Extant populations in the eastern Alps are diverse in mtDNA haplotypes, whereas populations in the western Alps are practically monomorphic (Gugerli et al. 2001b; Tollefsrud et al. 2008). These authors assume that one maternal lineage dominated during the postglacial colonization of the western Alps (founder event) and, once established and widespread, managed to remain in the western Alpine range despite recurrent population declines and recoveries (bottleneck effects). In analogy to such colonization dynamics, extant peripheral populations isolated from core populations, but also separated from each other, tend to show both low genetic diversity and high genetic differentiation (Hilfiker et al. 2004; Gapare et al. 2005; Aizawa et al. 2008; Eckert et al. 2008). In such usually small populations, potential inbreeding, i.e. selfing or mating among close relatives, would re-enforce these genetic patterns.

Swiss stone pine (*Pinus cembra*) is a tree species that is largely restricted to the timberline ecotone in the Alps and the Carpathians (Zoller 1991; inset in Fig. 1). The distribution of *P. cembra* in Switzerland, marking the middle part of the species' Alpine occurrence, is characterized by two main ranges in the east and the west, respectively. These two ranges are composed of central-alpine core areas, namely the Engadine and its surroundings in the east (Grisons), and the Penninic Alps (Valais) in the west (Rikli 1909; Fig. 1). In addition, there are disjunct and small peripheral populations associated to the two core areas in the respective northern Alpine areas (Vaud, Fribourg, Bern in the Northwest, Glarus and St. Gallen in the Northeast). These populations represent the latitudinal periphery of the species' Alpine range (Fig. 1) and may also be considered ecologically marginal (Zoller 1991). Further populations occur in-between the two main ranges in the middle part of the Swiss Alps (Fig. 1). Fossil pollen, retrieved from lake sediments or mires, suggest that *P. cembra* has re-immigrated from a refugial area in or near the southeastern Alps (Burga and Perret 1998). The few genetic studies on *P. cembra* are not conclusive on the species' phylogeography, mostly because sampling in the Alps was limited (Szmidt 1982; Politov et al. 1999; Belokon et al. 2005) or because

other questions were addressed (Gugerli et al. 2001a; Höhn et al. (2009).

Based on the above fossil evidence, we hypothesize that *P. cembra* migrated from the southern and/or southeastern Alps into its current Swiss range, resulting in a reduction in haplotypic diversity from east to west owing to (repeated) founder events and/or bottlenecks. Accordingly, peripheral northern Alpine populations are expected to be genetically depleted and highly differentiated. As an alternative scenario, the two main ranges were re-colonized from independent refugial areas. Given that the time to genetically diverge during isolation was sufficiently long and that gene flow between the two extant gene pools has not been too strong after re-colonization, the genetic differentiation between the two main ranges should be substantial.

Using cpDNA microsatellites to mirror historical gene flow by pollen and seed, we addressed the following questions: (1) Does chloroplast haplotype diversity decrease along the suggested east–west migration pathway of *P. cembra* in the Swiss Alps? (2) Alternatively, can we detect discontinuities in the genetic structure which coincide with the delimitation of the two main ranges of the species in Switzerland? (3) Do peripheral populations show lower genetic diversity and higher differentiation than central populations, owing to occasional colonization from respective core populations and genetic drift?

Our complementary data analyses show that cpDNA variation gradually declines from east to west, with no clear genetic separation of the two main ranges. This finding is in line with evidence from fossil pollen that *P. cembra* re-colonized the Swiss Alps from a refugial area in or near the eastern or southeastern periphery of the Alps.

Materials and methods

Study species

Swiss stone pine (*Pinus cembra* L.) is a closed-cone, five-needle pine of subgenus *Strobus* subsection *Cembrae* (Liston et al. 1999). The species' natural range expands across the European Alps and the Carpathian mountains (Zoller 1991; Tutin et al. 1993). In association with European larch (*Larix decidua*) and Norway Spruce (*Picea abies*), Swiss stone pine grows in the range of the timber line and often predominates in continental areas, referred to as inner-alpine valleys, such as the Valais or the Engadine in Switzerland (Rikli 1909). On the other hand, the sites of northern peripheral populations may be considered also ecologically marginal, owing to the relatively high level of precipitation in those areas. Swiss stone pine is wind-pollinated, presumably outcrossing, and depends mainly on the European nutcracker (*Nucifraga caryocatactes*) for seed dispersal (Mattes 1990). Therefore, clusters of stems may often be encountered as a consequence of seeds germinating from seed caches (Tomback et al. 1993).

Study sites and sampling

We sampled 39 natural populations throughout the Swiss range of *P. cembra* (Fig. 1). Fifteen population samples were taken from Gugerli et al. (2001a), collected in 1997 and 1998 but re-analyzed for this study, the additional locations were sampled in spring 2006. Needles were collected from 16 randomly selected tall trees from across local populations, respecting 30 m distance between sampled trees where

Fig. 1 Locations of the populations of *Pinus cembra* sampled in the Swiss Alps. For abbreviations see Table 1. Within the eastern (*circles*) and western (*squares*) ranges, sampling locations were characterized as core (*filled*) or peripheral (*open*) populations. *Grey triangles* indicate central/southern Alpine populations. *Grey* areas in the inset illustrate the species' natural range (distribution map courtesy to Euforgen, http://www.bioversity international.com/Networks/Euforgen)
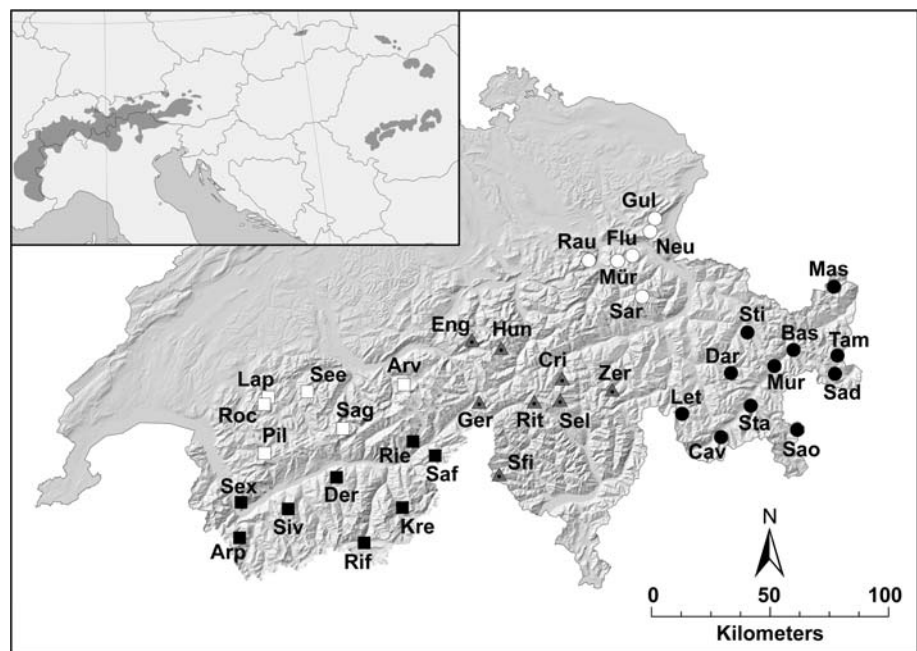
possible (total number of trees $N = 624$). We classified the sampling range into five subregions (Fig. 1) based on the biogeographical structuring of the Swiss stone pine range (Rikli 1909; Burga and Perret 1998). Eastern core ($n_{pop} = 11$) considered locations in and around the Engadine (Grisons), Eastern peripheral ($n_{pop} = 6$) included sites north/west of the (Vorder-)Rhein (Glarus, St. Galler Oberland, Churfirsten, Alpstein), Western core ($n_{pop} = 8$) comprised stands along the Rhone valley and its tributaries (Valais), and Western peripheral ($n_{pop} = 6$) combined locations along the northern slope of the main Alpine chain (Berner Oberland, Vaud, Fribourg). The Central/southern group ($n_{pop} = 8$) consisted of populations located outside of the two main ranges (central Switzerland, Ticino, western Grisons). This area was characterized by mostly disjunct occurrences of *P. cembra* with rather small census sizes. Core populations were large in area and census size as well as forming a dense network of occurrences, while peripheral populations were small, with only a few hundred individuals and clear spatial separation from neighboring populations.

Genetic analysis

As we presume that the plastid genome of *P. cembra* is strictly paternally inherited (M. Rüegg, unpublished data), cpDNA is dispersed by pollen and seed. We extracted genomic DNA using the DNeasy 96 plant kit (Qiagen, Hilden, Germany), following the manufacturers instructions. DNA concentrations were determined optically (Eppendorf Biophotometer; Vaudaux-Eppendorf, Schönenbuch, Switzerland). We amplified four mononucleotide cpDNA microsatellite loci (Pt15169, Pt26081, Pt36480, Pt63718; Vendramin et al. 1996). The 10 μL multiplex polymerase chain reaction (PCR) mix contained 1 μl of genomic DNA (1–2 ng/μL), 1× Multiplex PCR Mastermix (Qiagen), 1 mM additional $MgCl_2$ (final concentration of 4 mM), and 1 mM of primers. We used a mixture of four primer pairs, with one of the primers per pair fluorescently labeled (Pt15169: FAM; Pt26081: NED; Pt36480: HEX; Pt63718: FAM). PCR reactions were subjected to 95°C for 15 min to activate the HotStart DNA polymerase, followed by 23 cycles at 94°C for 30 s, 55°C for 90 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min, using a PTC-100 thermocycler (MJ Research, Waltham, USA). The lengths of amplification products were analyzed on an automated ABI 3100-Avant capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland) using ROX400HD (Applied Biosystems) as internal size standard. We ran a control individual on every 96-well plate to check for sizing consistency. Size variants were scored visually using GENEMAPPER 3.7 (Applied Biosystems). Haplotypes were denoted based on the size variant combination across the four cpSSR loci.

Statistical analysis

We tested for a simple Spearman rank correlation, with Bonferroni-corrected significance levels, between the populations' easting co-ordinates and their cpSSR diversity measured as Nei's haplotypic diversity or number of haplotypes, hypothesizing to find a gradual decrease in diversity along the putative re-colonization route from east to west. A Mantel matrix correlation was calculated to test for isolation by distance. We used Slatkin's (1995) linearized F_{ST}, $F_{ST}/(1 - F_{ST})$, as a measure of genetic distance between pairs of populations, and logarithmic Euclidean distance (in km) for geographic distance. Significance testing was based on 1,000 permutations in ARLEQUIN 3.11 (Excoffier et al. 2007). The partitioning of total genetic variation among and within populations was assessed in an analysis of molecular variance (AMOVA; ARLEQUIN 3.11).

To evaluate our alternative hypothesis of two genetically separated main ranges, we used two complementary approaches. First, we applied the software BARRIER 2.2 (Manni et al. 2004), which uses Monmonier's (1973) algorithm to identify discontinuities in genetic differentiation in relation to the populations' geographical arrangement. Thus, the largest value from a matrix of pairwise distances is taken as the starting segment of a particular barrier. It is then expanded in both directions, choosing the higher of the two values possible, until the margin or an existing barrier is met. Barriers may thus be seen as "genetic breaks" between adjacent singular or groups of populations. We established the Voronoï tessellation (with virtual external points) using the coordinates of the sampling locations, and calculated ten barriers based on maximum discontinuities in the matrix of pairwise F_{ST} values taken from ARLEQUIN 3.11. Slightly negative F_{ST} estimates, which are theoretically not defined and do not significantly differ from zero, were transformed to the respective positive values, since BARRIER does not accept negative distance values. Second, a spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) was applied to analyze population groupings. The software SAMOVA 1.0 searches for K groups of adjacent populations to maximize among-group genetic variation (F_{CT}). The underlying assumption is that maximum genetic differentiation among K groups best circumscribes spatially homogeneous population clusters. We tested for $K = 2$–10, selecting 100 random starting situations (for details see Dupanloup et al. 2002).

When testing for a difference of genetic diversity between core and peripheral populations, we applied a nested analysis of variance (ANOVA; core vs. peripheral nested within eastern vs. western) with Nei's (1973) haplotypic diversity as dependent variable using SYSTAT 10.0

(SPSS Inc. 2000). To evaluate the genetic differentiation among core and peripheral populations, respectively, we used again the transformed matrix of pairwise F_{ST} values from the ARLEQUIN output, considering only core–core and peripheral–peripheral population pairs within respective ranges. After data transformation [square-root of arcsine (F_{ST}) to account for the normality of the data], we performed a one-way ANOVA to test for a difference of mean F_{ST} values between these two groups. Because such pairwise data are non-independent, we applied a permutation test in ECOSIM (Gotelli and Entsminger 2008) to determine the statistical significance of the observed F ratio against the distribution of 1,000 iterated outcomes.

Results

We identified 3–9 size variants per cpSSR locus, and the totally 22 size variants at the four loci combined into 28 different haplotypes (Supplementary Table A). The dominant haplotype represented 60% of the total sample and was found in all study populations, while 13 rare haplotypes were population-specific (Table 1). In the overall AMOVA, 6.75% of total genetic variation (i.e. overall $F_{ST} = 0.068$; $P < 0.001$) was attributed to among-population differentiation, while the majority of variation was found within populations (93.25%; $P < 0.001$).

We identified a significant decrease in cpSSR diversity from east to west in both indices of genetic diversity tested (Nei's haplotypic diversity, $r = 0.597$, $P < 0.001$; number of haplotypes, $r = 0.460$, $P < 0.01$; Fig. 2). However, the matrix correlation between logarithmic geographic distance and genetic differentiation was low and not significant ($r = 0.032$, $P = 0.241$), rejecting the hypothesis of isolation by distance.

The first genetic discontinuity detected in BARRIER separated four eastern peripheral populations from the rest of the populations (Fig. 3). Of the following nine barriers, only the fourth represented a major grouping, separating seven of the eleven eastern core populations (Fig. 3). The remaining barriers were "erratic", separating one or two populations based on, with one exception, non-significant F_{ST} values (Fig. 3).

SAMOVA showed the highest regional differentiation at $K = 2$, with only one population (Hundsalp) separated from the remaining samples ($F_{CT} = 0.269$, $P < 0.05$; Supplementary Table B). When successively increasing group number until $K = 10$, mainly single populations were separated from the bulk group (Supplementary Table B).

Excluding central/southern populations that could not be unequivocally associated to either of the two main ranges of *P. cembra* in Switzerland, we found a significant difference in Nei's haplotypic diversity between core and peripheral populations (group means were 0.472 and 0.643, respectively; $P < 0.05$; Table 2). Mean pairwise F_{ST} values were significantly lower between core populations (0.185 ± 0.009) than between peripheral populations (0.283 ± 0.015) based on the permutation test implemented in ECOSIM ($F_{observed} = 26.4$, $P < 0.001$).

Discussion

In this study, we assessed the chloroplast haplotype structure in *Pinus cembra* populations of the Swiss Alps to infer historical processes mainly related to postglacial colonization dynamics. Our findings corroborate previous evidence that the species re-immigrated from a (south-)eastern Alpine refugial area, as inferred from fossil records. The gradual decrease in genetic diversity from east to west was additionally associated with reduced genetic diversity and strong differentiation of peripheral populations. Eckert et al. (2008), reviewing 134 studies on 115 species, reported that 64% of these studies found a decline in genetic diversity from core to peripheral populations. This pattern is thought to reflect the process of genetic drift through founder events and/or bottlenecks, coupled with reduced gene flow. Such a genetic structure is in accordance with the "abundant center model" (Brussard 1984; Lawton 1993). We consider these processes to underlie the diversity gradient from core to peripheral populations detected in *P. cembra* of the Swiss Alps.

Re-colonization from one or several refugial areas?

The occurrence of *P. cembra* in the Swiss Alps has been described in Rikli (1909) as forming two distinct main ranges, namely the eastern and western central Alps with core and respective peripheral populations. The core areas are characterized by a continental climate, giving a competitive advantage to *P. cembra* over Norway spruce, which is otherwise dominating the subalpine vegetation zone of the Alps. This biogeographical separation may be taken as an indication that *P. cembra* re-colonized the middle part of the Alps from an eastern and a western refugial area, respectively. However, evidence from fossil pollen suggests an East-to-West migration pathway (Burga and Perret 1998). The pattern of chloroplast haplotype diversity and differentiation described in this article supports the hypothesis of a single eastern refugial area, as we outline below.

Evidence for this conclusion comes from the gradual decline in genetic diversity from east to west (Fig. 2). Genetic diversity is expected to generally decrease along

Table 1 Study populations of *Pinus cembra* in the Swiss Alps

Region	Local name	Community	Elevation (m a.s.l.)	Haplotypes[a,b]
Eastern core	Alp **Sad**ra	Fuldera	2,000	A,B,C,D,G,I,L,**V**
	Darlux	Bergün	2,000	A,B,C,G,K
	God **Bas**elgia	Zernez	2,000	A,B,C,D,E,**ZC,ZD,ZE**
	God **Mur**ter	S-chanf	2,000	A,C,G
	God **Tam**angur	Scuol	2,150	A,C,D,F,G,J,M,**W**
	Lago da **Cav**loc	Stampa	1,900	A,B,C,D,E,J
	Letziwald	Avers	1,850	A,B,C,E,L,**ZA**
	Saoseo	Poschiavo	2,000	A,B,C,G,L,M,N,**Z,ZB,ZG**
	Stazerwald	Celerina	1,850	A,B,C,D,F,G,K
	Stillberg	Davos	1,950	A,B,C,E,G,I,J,L
	Val **Mas**auna	Samnaun	2,000	A,B,D,E,L,**R**
Eastern peripheral	**Flu**mserberge	Quarten	1,700	A,B,C
	Gulmen	Wildhaus	1,800	A,G,J,**Q**
	Mürtschenalp	Obstalden	1,650	A,D,E,G,K
	Neuenalp	Grabs	1,750	A,B,L,M
	Rautialp	Näfels	1,700	A,B,C,D,E
	Sardonaalp	Pfäfers	1,800	A
Western core	Fôret de **Der**bellec	Chandolins	2,050	A,C,G,J,N
	Kreuzboden	Saas Grund	2,200	A,C,K,L,O
	Riederalp	Ried-Mörel	1,950	A,B,K,M,P
	Riffelalp	Zermatt	2,000	A,D,G,**H**
	Saflischtal	Gregniols	2,000	A,B,D,L
	Sex Carro	Dorénaz	1,900	A,C,D,L
	Siviez	Nendaz	2,150	A,C,F,G,L
	Val d'**Arp**ette	Orsière	2,000	A,B,C,D,G
Western peripheral	**Arv**engarten	Grindelwald	1,900	A,D,G
	Col du **Pil**lon	Ormont Dessus	1,900	A,B,D,G,K,L,P
	Fôret du **Lap**é	Charmey	1,750	A,G,K
	Rochers des Rayes	Rougemont	1,700	A,D,G
	Sagiwald	Kandersteg	1,950	A,G,L
	Seeberg	Zweisimmen	1,800	A,G,**ZF**
Central/southern	Alpe di **Sfi**i	Campo	1,800	A,D,G,L
	Engstlenalp	Innertkirchen	1,900	A,B,C,G,L
	Geretal	Oberwald	1,900	A,B,D,G
	Hundsalp	Wassen	1,700	A,C,D,G,L,N
	Lago **Rit**om	Quinto	1,800	A,B,C
	Selva **Sec**ca	Olivone	1,800	A,B,C,G,J,L
	Val **Cri**stallina	Medel	1,700	A,B,C,D,
	Zervreila	Vals	1,900	A,B,C,E,G

Assignment of populations to regions, local name (with abbreviation in bold), community, approximate elevation, and occurring haplotypes

[a] Allele combinations of haplotypes are specified in Supplementary Table A

[b] Bold type indicates private haplotypes

colonization routes owing to genetic drift induced through founder events and bottleneck effects (Gugerli et al. 2001b). Accordingly, one should find a gradient from high to low genetic diversity with increasing distance to putative refugial areas. The consistent reduction in haplotypic diversity observed in *P. cembra* from east to west is in line with a continuous colonization from a putative eastern Alpine refugium. An additional major migration pathway, e.g., originating from a western or southern refugium, should otherwise be evident in a clear regional genetic
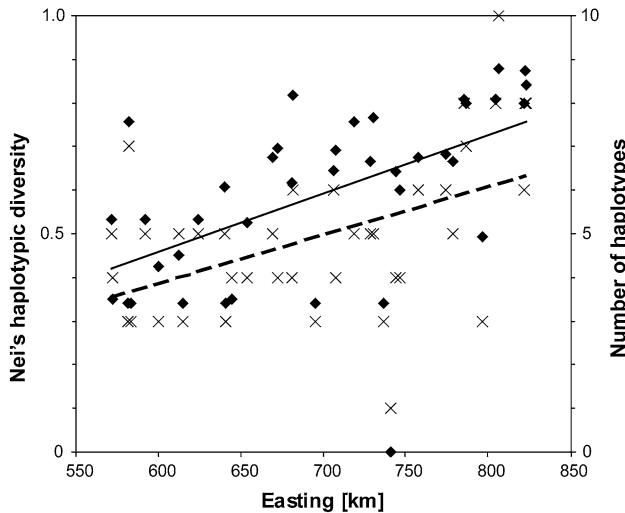
Fig. 2 Relationship between easting co-ordinates of 39 populations of *Pinus cembra* from the Swiss Alps and their chloroplast microsatellite diversity (Nei's (1973) haplotypic diversity: *diamonds, solid line*; number of haplotypes: *crosses, dashed line*)

Table 2 Results of the analysis of variance (ANOVA) to test for differences in genetic diversity of 31 Swiss populations of *Pinus cembra* based on variation in chloroplast DNA microsatellites

| Source of variation	df	MS	F
E versus W	1	0.215	1.163
C versus P (nested)	2	0.185	4.126*
Error	27	0.045	

Nei's gene diversity was analyzed in a nested design (core vs. peripheral populations nested within eastern vs. western populations)

C Core, *P* peripheral, *E* eastern, *W* western populations

* $P < 0.05$

differentiation (see below). In accordance with the above scenario, we found high cpDNA polymorphisms in two populations from Austria, one population from Germany (unpublished data), and other eastern Alpine populations assessed with only three cpDNA SSR loci (Vendramin et al., unpublished data).

We further consider the distribution of rare private haplotypes as an argument against an additional western refugium, as eleven out of 13 private haplotypes occurred in eastern populations, while only two haplotypes were private, one each for a western core and a western peripheral population, respectively (Table 1). These private haplotypes may reflect either occasional local

mutations or gene flow from otherwise unsampled populations. We would expect western or southern populations to harbor high-frequency private haplotypes if another refugium was of relevance for the pattern of extant cpDNA structure.

In our scenario, we assume that the peripheral populations were colonized from the respective core areas. An alternative hypothesis could be that western populations per se were peripheral given a westward migration route. However, we consider this scenario as less likely than the one we propose. Both eastern and western Swiss ranges are characterized by core areas in which *P. cembra* is widespread and dominant in the subalpine forest ecosystems. These areas also represent the core of the species' natural range and reflect the species' ecological optimum. Our analyses do not allow us to infer the exact migration route of *P. cembra* into its western Alpine range. Parisod (2008) argues that a direct immigration from eastern refugia (transalpine eastern pathway) played a marginal role in the postglacial colonization of the Valais as compared to alternative routes

Fig. 3 Identification of genetic discontinuities (barriers) among the 39 populations of *Pinus cembra* from the Swiss Alps. The fine *black line* polygons around population centers delineate the Voronoï tesselation as established in BARRIER 2.2 (Manni et al. 2004). *Thick lines* represent the ten most relevant genetic discontinuities based on the matrix of pairwise F_{ST} values. Barrier importance decreases from *black* to *light grey*, with *solid lines* representing stronger barriers than *dashed lines* of the same tone. For population abbreviations see Table 1, for symbols see Fig. 1
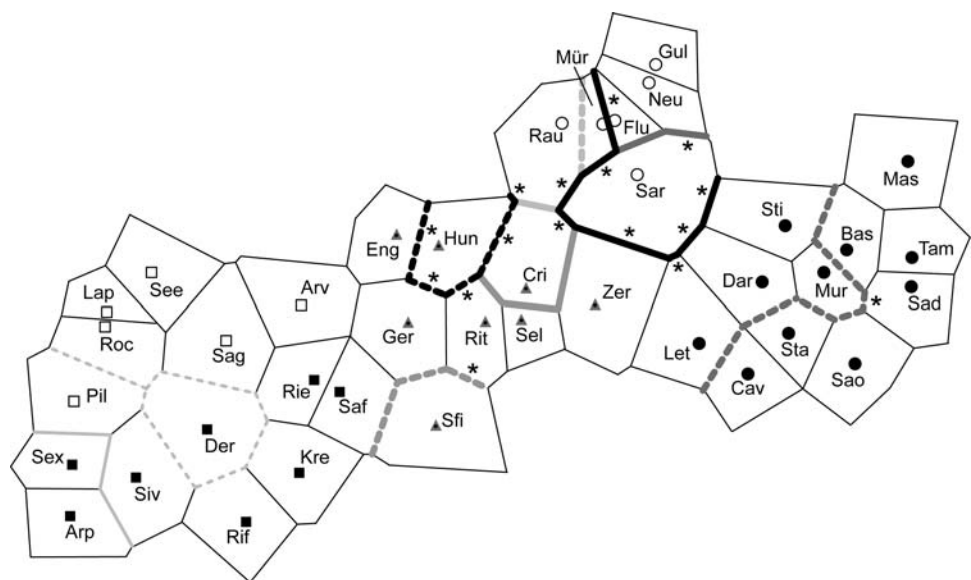
from the south or the west. While *P. cembra* might have colonized the Valais by circumventing the southern Alps and migrating into the Valais from the West, we also note that the species is mainly dispersed by the European nutcracker, a bird capable of flying across large distances carrying stone pine seeds (Mattes 1990). A passage across the low passes between eastern and western core ranges would be a plausible hypothesis.

Overall, the pattern of genetic differentiation was rather shallow, as seen from the low partition of among-population variation in the AMOVA ($F_{ST} = 0.068$) as well as from the SAMOVA (Supplementary Table B) and the BARRIER results (Fig. 3). Also the non-significant Mantel correlation, i.e. no isolation by distance, fits into this pattern. Such a genetic structure could be explained by recent expansion, which to a certain degree reflects the relatively low number of generations that have passed since the immigration of *P. cembra* in the Swiss Alps. On the other hand, cpDNA is primarily dispersed by pollen, so that substantial gene flow among populations might also partly account for the low degree of genetic differentiation.

Extensive historical gene flow is also evident over the species' natural range. There is only little genetic differentiation between *P. cembra* populations from the Alps and the Carpathians (3% of the total genetic variation; Höhn et al. 2009). It is likely that the populations of *P. cembra* in the two mountain ranges are descendents from a common gene pool that formerly occupied a larger range. In the course of climate warming after the last glacial maximum, the range contracted in the Carpathians, while in the Alps, it expanded westwards into the formerly glaciated area. Random sampling effects such as genetic drift, founder events, or bottlenecks have consequently occurred and are still evident in the extant pattern of genetic diversity.

Genetic drift in extant peripheral populations?

We expected to find low cpDNA diversity in peripheral as compared to core populations of *P. cembra* as an effect of genetic drift and occasional colonization. Our results clearly showed a significantly lower gene diversity and a higher genetic differentiation of northern Alpine compared to core populations (Table 2). Both results conform to the "abundant center model" (Brussard 1984; Lawton 1993).

The shallow structure of cpDNA variation detected in *P. cembra* in the Swiss Alps contrasts with the distinct bottleneck effect inferred from the pattern of mtDNA haplotype distribution of *Picea abies* across the Alps (Gugerli et al. 2001b; Tollefsrud et al. 2008). However, the

difference may be explained by the dispersal mode of the two organellar genomes. Mitochondria are maternally inherited and, thus, only dispersed via seed, while paternally inherited cpDNA markers are spread through both pollen and seed. Since dispersal distances of the light pollen are likely to exceed those of the heavier seed in *P. abies* and even more so in *P. cembra*, the genetic structure resulting from pollen- and seed-mediated gene flow should be less pronounced. Indeed, cpDNA differentiation in *P. abies* is less distinct than mtDNA in the Alps (Bucci and Vendramin 2000; Gugerli et al. 2002). A similar situation displaying distinct mtDNA separation versus fuzzy cpDNA haplotype distribution was described for Silver fir (*Abies alba*) by Liepelt et al. (2002).

In a study of *P. cembra* populations from the Carpathians and the Alps (Höhn et al. 2009), the latter represented by a subset of the populations analyzed in the present study, population differentiation was substantially greater in the Carpathians than in the Alps. Höhn et al. (2009) explained the higher genetic differentiation among the rather fragmented occurrences of *P. cembra* in the Carpathians as the result of spatial isolation hampering gene flow.

In summary, the larger, continuous, and more abundant populations of the core area of *P. cembra* in the Swiss Alps have experienced less genetic drift and more frequent pollen and/or seed gene flow as compared to the isolated peripheral populations, which contributed to their retaining larger haplotypic diversity. This is not to say that cpDNA haplotype diversity directly affects the local populations' plasticity or adaptation to particular habitat types. However, more suitable habitat, both in terms of habitat potential and available area, as in the core range of *P. cembra*, offers higher chances for cpDNA haplotypes to immigrate through successful fertilization (pollen) or germination/establishment (seed) (Zimmermann and Gugerli 2005). However, this trend awaits more detailed investigation, both in a historical context and related to upward shifts of the timberline ecotone as currently observed (Gehrig-Fasel et al. 2007). We also anticipate that small-scale processes may differ among populations varying in size and degree of isolation. An on-going analysis of nuclear markers (Salzer et al. 2009) in combination with germination and common-garden experiments will reveal whether within-population processes such as potential inbreeding and fitness reduction may be relevant for the conservation of small remnant populations of *P. cembra*.

References

Aizawa M, Yoshimaru H, Katsuki T, Kaji M (2008) Imprint of post-glacial history in a narrowly distributed endemic spruce, *Picea alcoquiana*, in central Japan observed in nuclear microsatellites and organelle DNA markers. J Biogeogr 35:1295–1307

Avise JC (2009) Phylogeography: retrospect and prospect. J Biogeogr 36:3–15

Belokon MM, Belokon YS, Politov DV, Altukhov YP (2005) Allozyme polymorphism of Swiss stone pine *Pinus cembra* L. in mountain populations of the Alps and the Eastern Carpathians. Russ J Genet 41:1268–1280

Brewer S, Cheddadi R, de Beaulieu J-L, Reille M, Data Contributors (2002) The spread of deciduous *Quercus* throughout Europe since the last glacial period. For Ecol Manage 156:27–48

Brussard PF (1984) Geographic patterns and environmental gradients: the central–marginal model in *Drosophila* revisited. Annu Rev Ecol Syst 15:25–64

Bucci G, Vendramin GG (2000) Delineation of genetic zones in the European Norway spruce natural range: preliminary evidence. Mol Ecol 9:923–934

Burga CA, Perret R (1998) Vegetation und Klima der Schweiz seit dem jüngeren Eiszeitalter. Ott, Thun

Cheddadi R, Vendramin GG, Litt T, François L, Kageyama M, Lorentz S, Laurent J-M, de Beaulieu J-L, Sadori L, Jost A, Lunt D (2006) Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. Glob Ecol Biogeogr 15:271–282

Comps B, Gömöry D, Letouzey J, Thiébaut B, Petit RJ (2001) Diverging trends between heterozygosity and allelic richness during postglacial colonization in the European beech. Genetics 157:389–397

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. Mol Ecol 11:2571–2581

Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central–marginal hypothesis and beyond. Mol Ecol 17:1170–1188

Excoffier L, Laval G, Schneider S (2007) Arlequin: a software for population genetic data analysis. Computational and Molecular Population Genetics Lab, University of Bern, Bern

Gapare WJ, Aitken SN, Ritland CE (2005) Genetic diversity of core and peripheral Sitka spruce [*Picea sitchensis* (Bong.) Carr] populations: implications for conservation of widespread species. Biol Conserv 123:113–123

Gehrig-Fasel J, Guisan A, Zimmermann NE (2007) Tree line shifts in the Swiss Alps: climate change or land abandonment? J Veg Sci 18:571–582

Gitzendanner MA, Soltis PS (2000) Patterns of genetic variation in rare and widespread plant congeners. Am J Bot 87:783–792

Gotelli NJ, Entsminger GL (2008) EcoSim: null models software for ecology. Acquired Intelligence and Kesey-Bear, Jericho

Gugerli F, Senn J, Anzidei M, Madaghiele A, Büchler U, Sperisen C, Vendramin GG (2001a) Chloroplast microsatellites and mitochondrial *nad*1 intron 2 sequences indicate congruent phylogeographic relationship of Swiss stone pine (*Pinus cembra*), Siberian stone pine (*P. sibirica*), and Siberian dwarf pine (*P. pumila*). Mol Ecol 10:1489–1497

Gugerli F, Sperisen C, Büchler U, Magni F, Geburek T, Jeandroz S, Senn J (2001b) Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. Mol Ecol 10:1255–1263

Gugerli F, Sperisen C, Mátyás G, Senn J, Anzidei M, Vendramin GG (2002) Molecular markers help to reveal postglacial

re-colonisation routes of forest tree species in the Alpine region. In: Bottarin R, Tappeiner U (eds) Interdisciplinary mountain research. Blackwell, Berlin, pp 156–161

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58:247–276

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Phil Trans R Soc Lond B 359:183–195

Hilfiker K, Gugerli F, Schütz J-P, Rotach P, Holderegger R (2004) Low RAPD variation and female-biased sex ratio indicate genetic drift in small populations of the dioecious conifer *Taxus baccata* in Switzerland. Conserv Genet 5:357–365

Höhn M, Gugerli F, Abran P, Bisztray G, Buonamici A, Cseke K, Hufnagel L, Sebastiani F, Quintela-Sabarís C, Vendramin GG (2009) Variation in the chloroplast DNA of Swiss stone pine (*Pinus cembra* L.) reflects contrasting post-glacial history of populations from the Carpathians and the Alps. J Biogeogr. doi:10.1111/j.1365-2699.2009.02122.x

Lawton JH (1993) Range, population abundance and conservation. Trends Ecol Evol 8:409–413

Liepelt S, Bialozyt R, Ziegenhagen B (2002) Wind-dispersed pollen mediates postglacial gene flow among refugia. Proc Natl Acad Sci USA 99:14590–14594

Liston A, Robinson WA, Piñero D, Alvarez-Buylla ER (1999) Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Mol Phylogenet Evol 11:95–109

Magri D, Vendramin GG, Comps B, Dupanloup I, Geburek T, Gömöry D, Latalowa M, Litt T, Paule L, Roure JM, Tantau I, van der Knaap WO, Petit RJ, de Beaulieu J-L (2006) A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. New Phytol 171:199–221

Manni F, Guérard E, Heyer E (2004) Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by "Monmonier's algorithm". Hum Biol 76:173–190

Mattes H (1990) Die Lebensgemeinschaft von Tannenhäher und Arve. Eidgenössische Forschungsanstalt für das forstliche Versuchswesen, Birmensdorf

Monmonier MS (1973) Maximum-difference barriers: an alternative numerical regionalization method. Geogr Anal 5:245–261

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323

Parisod C (2008) Postglacial recolonisation of plants in the western Alps of Switzerland. Bot Helv 118:1–12

Petit R, Vendramin GG (2007) Plant phylogeography based on organelle genes: an introduction. In: Weiss S, Ferrand N (eds) Phylogeography of Southern European refugia—Evolutionary perspectives on the origins and conservation of European biodiversity. Springer, Dordrecht, pp 23–97

Petit RJ, Csaikl UM, Bordács S, Burg K, Coart E, Cottrell J, van Dam B, Deans JD, Dumolin-Lapègue S, Fineschi S, Finkeldey R, Gillies A, Glaz I, Goicoechea PG, Jensen JS, König AO, Lowe AJ, Madsen SF, Mátyás G, Munro RC, Olalde M, Pemonge M-H, Popescu F, Slade D, Tabbener H, Taurichini D, de Vries SGM, Ziegenhagen B, Kremer A (2002) Chloroplast DNA variation in European white oaks: phylogeography and patterns of diversity based on data from over 2600 populations. For Ecol Manage 156:5–26

Petit RJ, Aguinagalde I, de Beaulieu J-L, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Müller-Starck G, Demesure-Musch B, Palmé A, Martín JP, Rendell S, Vendramin GG (2003) Glacial refugia:

hotspots but not melting pots of genetic diversity. Science 300:1563–1565

Petit RJ, Bialozyt R, Garnier-Gere P, Hampe A (2004) Ecology and genetics of tree invasions: from recent introductions to Quaternary migrations. For Ecol Manage 197:117–137

Politov DV, Belokon MM, Maluchenko OP, Belokon YS, Molozhnikov VN, Mejnartowicz LE, Krutovskii KV (1999) Genetic evidence of natural hybridization between Siberian stone pine, *Pinus sibirica* Du Tour, and dwarf Siberian pine, *P. pumila* (Pall.) Regel. For Genet 6:41–48

Rikli M (1909) Die Arve in der Schweiz Neue Denkschr Schweiz Naturforsch Ges 44:1–455

Salzer K, Sebastiani F, Gugerli F, Buonamici A, Vendramin GG (2009) Isolation and characterization of eight polymorphic nuclear microsatellite loci in *Pinus cembra* L. Mol Ecol Res 9:858–861

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462

Soltis DE, Morris AB, McLachlan JS, Manos PS, Soltis PS (2006) Comparative phylogeography of unglaciated eastern North America. Mol Ecol 15:4261–4293

Szmidt AE (1982) Genetic variation in isolated populations of Stone Pine (*Pinus cembra*). Silva Fenn 16:196–200

Tollefsrud MM, Kissling R, Gugerli F, Johnsen Ø, Skroppa T, Cheddadi R, van der Knaap P, Latalowa M, Terhürne-Berson R, Litt T, Geburek T, Brochmann C, Sperisen C (2008) Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. Mol Ecol 17:3134–3150

Tomback DF, Holtmeier F-K, Mattes H, Carsey K, Powell ML (1993) Tree clusters and growth form distribution in *Pinus cembra*, a bird-dispersed pine. Arctic Alpine Res 25:374–381

Tribsch A (2004) Areas of endemism of vascular plants in the Eastern Alps in relation to Pleistocene glaciation. J Biogeogr 31:747–760

Tutin TG, Burges NA, Chater AO, Edmonds JR, Heywood VH, Moore DM, Valentine DH, Walters SM, Webb DA (eds) (1993) Flora Europaea. Cambridge University Press, Cambridge

Vendramin GG, Lelli L, Rossi P, Morgante M (1996) A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. Mol Ecol 5:595–598

Zimmermann N, Gugerli F (2005) Linking intra-specific diversity and habitat suitability—a case study using *Pinus cembra* L. In: Proceedings of the International Botanical Congress, Vienna, July 17–23, p 212

Zoller H (1991) *Pinus*. In: Conert HJ, Hamann U, Schultze-Motel W, Wagenitz G (eds) Gustav Hegi—Illustrierte Flora von Mitteleuropa. Blackwell, Berlin, pp 77–83