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Reduced fitness at early life stages in peripheral versus core populations of Swiss stone pine (*Pinus cembra*) is not reflected by levels of inbreeding in seed families

Kristina Salzer · Felix Gugerli

Abstract Small and fragmented populations are prone to mating among related individuals, increasing homozygosity and likely negatively affecting offspring fitness. Such a trend may be enforced by environmental changes in species with narrow ecological niches because inbred populations are more prone to become maladapted as compared to outcrossed populations. Here, we studied differences in offspring fitness and inbreeding levels between core and peripheral populations of Swiss stone pine (*Pinus cembra*). We collected open-pollinated progenies of *P. cembra* in large, contiguous and in small, isolated populations (core vs. periphery). Seeds were germinated and grown in a common garden to test for differences in fitness parameters, whereas subsamples of seed lots were genotyped at seven nuclear microsatellites to calculate selfing rates. We found significantly lower seed production, higher embryo abortion rates and lower germination success in small peripheral compared to large core populations of *P. cembra*. In contrast, winter survival and first-year growth of seedlings did not significantly differ between the two population types. These results indicate higher inbreeding depression at the earliest life stages in small and fragmented populations compared to populations from the contiguous range of *P. cembra*. However, we found no correlation between any fitness parameters and progeny-derived selfing rates. We explain this discrepancy by the fact that inbreeding depression mostly affects embryo abortion rates in Pinaceae. This cannot be genetically quantified because non-developed embryos cannot be genotyped. We infer that population fragmentation in the long term negatively affects natural regeneration in this long-lived, wind-pollinated conifer species.

Keywords Common garden experiment · Core/periphery · Germination · Offspring fitness · Selfing rate · Small population size

Introduction

Small population size and spatial isolation can promote consanguineous mating in natural populations, promoting excess homozygote frequencies in subsequent generations (Allendorf and Luikart 2007; Mimura and Aitken 2007). Likewise, spatial aggregation of related individuals may lead to increased levels of homozygosity in offspring and the possibility that recessive deleterious or lethal alleles are expressed (Charlesworth and Charlesworth 1987; Keller and Waller 2002). Consequently, inbred progeny may show inbreeding depression, i.e. reduced fitness compared to outbred offspring. Inbreeding in predominantly outcrossing conifers has been shown to have strong impacts on the fitness of progeny (e.g. Sorensen 1969; Sorensen and Miles 1982), particularly at early life stages, due to the high genetic load typical for conifers (Namkoong and Bishir 1987; Savolainen et al. 1992). In Pinaceae, inbreeding depression generally results in higher embryo abortion, lower germination success, reduced growth, survival and fecundity when compared to non-inbred individuals (Lande and Schemske 1985; Kärkkäinen and Savolainen 1993; Husband and Schemske 1996; Kärkkäinen et al. 1999). In self-compatible

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plants such as conifers, homozygosity can also arise through self-fertilization. Accordingly, self-fertilized progeny of Scots pine (*Pinus sylvestris*), showed significant inbreeding depression in seed maturation and post-germination survival (Kärkkäinen et al. 1999; Koelewijn et al. 1999). In addition, a study on reproductive fitness traits and mating system in eastern white pine (*Pinus strobus*) revealed significantly lower reproductive fitness in small and marginal stands compared to large and central populations (Rajora et al. 2002).

Together with other factors like genetic drift and reduced gene flow, inbreeding in small populations may hamper population persistence in the long term (Ellstrand and Elam 1993; Keller and Waller 2002). At the same time, inbreeding may mitigate its negative effects due to selection against inbred offspring, thus, purging deleterious alleles (Lande and Schemske 1985; Barrett and Charlesworth 1991; Husband and Schemske 1996; Sorensen 2001). In *P. sylvestris*, for instance, populations in northern Finland revealed lower inbreeding depression than populations in the South. Hedrick et al. (1999) proposed that these differences might be explained with stronger selection against inbred individuals in northern compared to southern stands. In lodgepole pine (*Pinus contorta*), higher selfing rates partially reduced the negative effects of inbreeding on offspring (Sorensen 2001). However, other findings suggested that purging may lower population viability through the fixation of mildly deleterious alleles (e.g. Leberg and Firmin 2008 and references therein).

Swiss stone pine (*Pinus cembra*) is a European conifer species of particular interest, as it grows in high-elevated mountain areas, at subalpine altitudes at or close to the timberline, often in association with Norway spruce (*Picea abies*) and European larch (*Larix decidua*; Rikli 1909; Zoller 1991; Tutin et al. 1993). Global warming is likely to affect this species, making it more susceptible to interspecific competition (Casalegno et al. 2010). In fact, a climate-driven negative population trend has been proposed to explain the decline of *P. cembra* during the past 8,000 years (Furrer 1955). In Switzerland, extensive anthropogenic exploitation of *P. cembra* has furthermore led to fragmented and small stands in the northern periphery of the Swiss Alps (Furrer 1955). In contrast, *P. cembra* still forms contiguous and large populations in the South and East of the central Alps in Switzerland, where the climate is continental and thus less suitable for *Picea abies* as the primary competitive tree species (Furrer 1955).

Moreover, *P. cembra* has a distinct dispersal ecology. The wind-pollinated, wingless seeds are largely dispersed by the European nutcracker (*Nucifraga caryocatactes*; Mattes 1990; Tomback et al. 1993). The corvid bird buries groups of 5–12 *P. cembra* seeds as a winter food resource. Unrecovered seeds may germinate, which often results in multi-stem tree clusters (Tomback et al. 1993). Accordingly, a molecular analysis suggested that spatially aggregated individuals

are often genetically related (Rüegg 2006). Moreover, strong spatial autocorrelation in an intensively studied population in the northern Swiss Alps confirmed the aggregation of genetically similar individuals potentially fostering mating among relatives (Salzer 2011). Hence, *P. cembra* is a well-suited species to study the effects of potential inbreeding, population fragmentation and small population size on the fitness of offspring.

Although genetic studies on the breeding system of *P. cembra* have found that outcrossing was predominant, outcrossing rates were lower than in other pine species (Krutovskii et al. 1995; Lewandowski and Burczyk 2000; Belokon et al. 2005; Politov et al. 2008). The relevance of these results is, however, difficult to evaluate, as the realized mating system in natural populations and the magnitude of early inbreeding depression have not been examined to date.

In order to better understand the effects of potential inbreeding in natural populations of *P. cembra*, we experimentally and genetically investigated open-pollinated progenies collected in two population types differing in size and in the degree of fragmentation. In doing so, we assume that averaged differences in offspring fitness in a common garden reflect the realized mating system in the respective population. Specifically, we hypothesized higher inbreeding levels and lower fitness performance in offspring from small and fragmented peripheral compared to respective parameters from large and contiguous core populations, which would be especially important in the light of global change and related increasing interspecific competition predicted for *P. cembra* (Casalegno et al. 2010). The direct comparison between experimentally assessed fitness and offspring-derived estimates of inbreeding of the same progenies, using molecular markers, will provide insights to the detectability and relevance of inbreeding in small, fragmented populations of a long-lived tree species.

Materials and methods

Study species and sampling locations

Pinus cembra L. is a monoecious closed-cone pine of the five-needle subsection *Cembrae* (Liston et al. 1999) in the subgenus *Strobus*. Its natural range expands across the European Alps and the Carpathian mountains (Zoller 1991; Tutin et al. 1993; Fig. 1). In Switzerland, the present distribution of *P. cembra* consists of two rather distinct ranges: while the species covers large and contiguous areas of the timberline ecotone in the continental central Alps, populations in the northern periphery of the Alps are much smaller and more fragmented (Rikli 1909). These disjunct occurrences are furthermore characterized by lower haplotypic diversity and higher genetic differentiation at chloroplast

Fig. 1 Locations of *Pinus cembra* populations sampled in the Swiss Alps (for abbreviations see Table 1). *Open circles* indicate peripheral populations and *filled circles* display core populations. *Grey areas* in the *inset* illustrate the species' natural distribution range (distribution map courtesy to Euforgen, http://www.euforgen.org/distribution_maps.html)
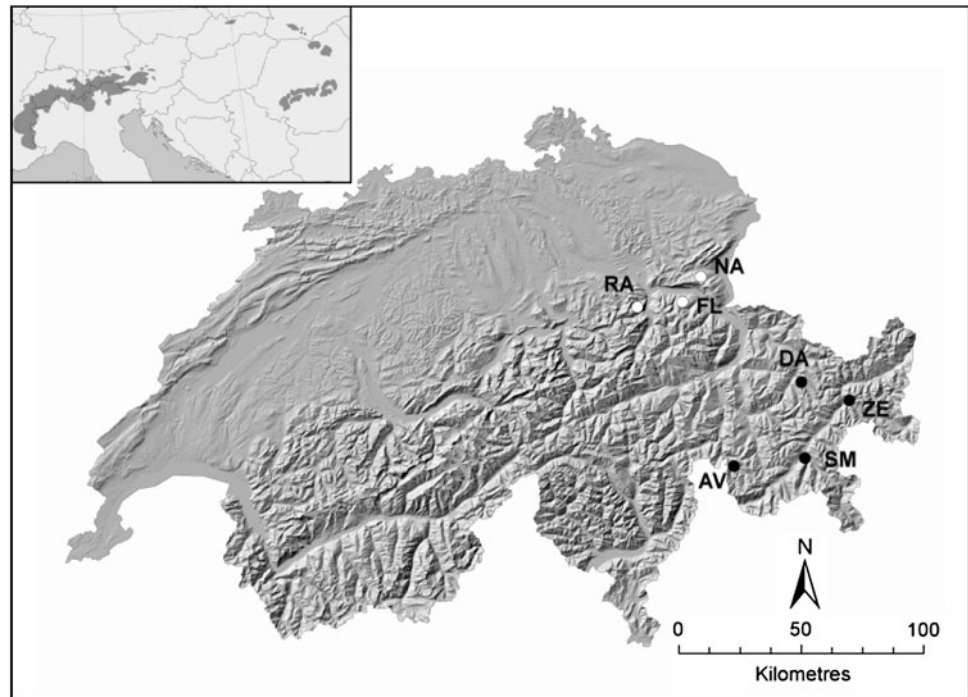
microsatellites (Gugerli et al. 2009). Based on the genetic structure detected, a common ancestry of the Swiss *P. cembra* populations from a single glacial refugium can be assumed (Gugerli et al. 2009). Hence, we classified our sampling locations into two major population types: large core populations, located in the contiguous range of the central Alps, versus small and fragmented populations in the northern range of the Alps, representing the periphery of the species' distribution range in the European Alps.

Given the generally low level of fruiting in the sampling season 2007, we obtained 4–25 open-pollinated cones of 3–11 maternal trees ($N = 543$) from 7 *P. cembra* stands, all located in eastern Switzerland: altogether, 38 trees could be sampled in 4 core populations and 16 trees in 3 peripheral populations (Table 1, Fig. 1). Trees sampled were at least 30 m distant from each other, and only tall single-stem trees were considered. Needle samples of seed trees were also collected to assess maternal genotypes.

Parental seed production and offspring performance

Cones were kept in humid sand for 3 months. Once the cones were dried and shattered, seeds were counted. In *Pinus*, a lack of pollination results in the abortion of ovules in the first year of seed development (Sarvas 1962). Such undeveloped seeds are easy to distinguish from those that were fertilized, due to their small size and scale-like shape. Therefore, we counted fully developed seeds as well as these first-year aborted ovules per cone. The latter were discarded while sets of fully developed seeds were divided in halves

Table 1 Sampling and estimates of inbreeding for offspring and adult *Pinus cembra* trees from core and peripheral populations in the eastern Swiss Alps

Population (Code)	$N_{families}$	N_{cones}	$N_{embryos}$	Estimates of inbreeding	
				$s_{offspring}$	$F_{IS\ adult}$
Core					
Zernez (ZE)	10	131	121	0.117	0.074
St Moritz (SM)	11	98	121	0.141	−0.029
Avers (AV)	7	67	105	0.063[a]	−0.002
Davos (DA)	10	91	120	0.052	0.109
Peripheral					
Rautialp (RA)	8	82	94	0.141	0.056
Neuenalp (NA)	5	47	88	0.562	0.128
Flumserberge (FL)	3	27	2	NA	0.124

Number of families ($N_{families}$), number of cones (N_{cones}) sampled, number of embryos genotyped ($N_{embryos}$) per population. Inbreeding was estimated as selfing ($s = 1 - t_m$) in offspring and F_{IS} in adults. F_{IS} was calculated from independent samples collected in the same populations (Gugerli et al. unpubl. data)

NA not available owing to insufficient number of embryos for genotyping

[a] t_m not significantly different from 1, indicating complete outcrossing

per cone. One half was dried at room temperature for laboratory analyses and the other half was subjected to a germination experiment. Note that these fully developed seeds comprised both empty and filled seeds (Table 2), and

we accounted for the proportion of filled seeds in the statistical model.

Seeds were stored in a peat/sand mixture in plastic bags and subjected to a cold/warm treatment during winter 2007/2008. With appearance of the first radicles in March 2008, seeds were sown in fertilized (4 g Rhodo fertilizer per l of substrate) peat/wood fibre substrate (4:1) in pots of 12 cm diameter. We evenly spread all seeds that were available per cone in one pot. Pots were repeatedly randomized and transferred from the greenhouse to a nursery at WSL Birmensdorf (47.36°N, 8.46°E; 550 m a.s.l.) after 6 weeks. Given the lowland location of the nursery, the local climate conditions substantially differed from what *P. cembra* experiences in its natural range in the Swiss Alps. At the nursery, monthly temperature averages were at 7.1 °C (mean; minimum −0.4 °C, maximum 15.5 °C) in 2008 and at 7.0 °C (−3.4/15.8 °C) in 2009, and annual precipitation summed up to 1145.5 mm in 2008 and 1112.8 mm in 2009. We buried the pots in peat substrate, watered them when necessary and covered them with a sun protection mat during the first 4 weeks, giving 50 % protection. We monitored germination during the vegetation period 2008. Per pot, we counted newly emerged as well as dead seedlings and determined the number of surviving seedlings in autumn 2008. All pots were maintained in the nursery during winter 2008/2009 without protection. After assessing seedling winter mortality in April 2009, we randomly chose five pots from all maternal trees, as far as seedlings were available, to examine the following parameters for two randomly chosen seedlings ($N = 404$, 45 seed families): (1) shoot length, i.e. distance between the first needle circle and the first horizontal root, (2) root length, i.e. distance between the first horizontal root and the root tip, (3) number of buds on apical shoot, and (4) needle length, i.e. length of the longest needle in the first needle circle.

Genetic analyses

We determined the average weight of dried, fully developed seeds per cone. We then randomly chose five cones from eight maternal trees per stand and excised embryos from three seeds per cone if at all available ($N = 651$; Table 1). After removing and discarding the woody outer part of the seeds, we soaked them in distilled water for 2 h to allow easy preparation of embryos. The total number of fully developed seeds that we had to prepare for obtaining 15 developed embryos per mother tree was later used to calculate a proportion value of filled seeds per individual (Table 2). DNAs from embryos and also needles were isolated with the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Nuclear microsatellite (nSSR) analysis followed the protocol published by Salzer et al. (2009). Locus Pc3 was excluded from the analysis due to difficulties in amplification. Linkage disequilibrium among microsatellite loci was tested with the programme FSTAT 2.9.3 (Goudet 1995). No significances were detected, thus, we included all seven loci in the analysis. All offspring genotypes were cross-checked with the respective maternal genotypes to avoid bias owing to genotyping error and/or null alleles.

Data analysis

We calculated fitness values (FVs) at four levels to compare reproductive output and offspring performance between population types: parental seed production, germination success, seedling survival and seedling growth (Table 2). For seed production and seedling growth, which both are parameters not resulting in proportional data per se, we calculated relative performance coefficients (RPCs): values (average value in case of growth data) obtained per cone were divided by the respective maximum (average) value among all cones. Regarding seed production, this allowed us to calculate one fitness value, comprising data on the parental contribution to the offspring, as a multiplicative function of RPCs (Table 2).

Germination success per cone was calculated on the basis of fully developed seeds per cone, which had been adjusted for the average percentage of filled seeds (i.e. fully developed seeds containing an embryo) per maternal tree

Table 2 Calculation design of fitness values (FV) as functions of one or several variables, including relative performance coefficients (RPCs), measured for *Pinus cembra* offspring from populations in the eastern Swiss Alps

	Variables included in FV
FV parental seed production	RPC total number of seeds × RPC number of fully developed seeds × RPC average weight of dried fully developed seed
FV germination success	Total number of emerged seedlings/total number of seeds × average proportion of filled seeds among fully developed seeds per maternal tree
FV seedling survival	Germination survival × winter survival
FV seedling first-year growth	RPC average needle length

(Table 2). Seedling survival comprised the seedling survival percentages during the first vegetation period and the first winter season. Regarding growth data, we first investigated correlations between all morphometric parameters measured using Spearman's rank correlations in SPSS 17.0 (SPSS 2008). Needle length correlated significantly with all other variables at $r_S > 0.2$ (needle–shoot: $r_S = 0.248$; needle–number of buds: $r_S = 0.316$; needle–seedling length: $r_S = 0.410$; $P < 0.01$ in all cases). Furthermore, we failed to detect a density effect of seedlings on needle length despite large variation in the number of emerged seedlings per pot. Consequently, we considered needle length as representative of first-year growth and used only these values in further analyses.

The requirements of normal distribution and homoscedasticity of data were met based on residual distributions after arcsine square root transformation of FVs. This allowed us to apply mixed hierarchical General Linear Models (GLMs), with maternal trees nested within populations, nested within population types. The latter was determined as fixed effect, maternal tree and population as random effects.

In order to account for relationships among variables in analyses of variance, we additionally examined correlations among all response variables that we investigated. Germination success was positively correlated with average weight of fully developed seeds ($r_{s\ (core)} = 0.502$, $P < 0.0001$, $N = 387$; $r_{s\ (peripheral)}$ 0.225, $P < 0.01$, $N = 135$). Seed weight was also positively correlated with seedling survival ($r_{s\ (core)} = 0.327$, $P < 0.0001$, $N = 341$; $r_{s\ (peripheral)} = 0.333$, $P = 0.078$, $N = 29$) and first-year growth in both population types ($r_{s\ (core)} = 0.632$, $P < 0.0001$, $N = 178$; $r_{s\ (peripheral)}$ 0.327, $P = 0.110$, $N = 25$), though only significantly so in core populations. There was no relevant relationship between seedling density and seedling survival, and we failed to detect a trade-off between total number of seeds and seed weight. Accordingly, we included only seed weight as a covariate in the respective GLMs. As the correlations were inconsistent between population types, we additionally incorporated the interaction between covariate and population type into analysis.

Due to the unbalanced sampling design, we applied the recommended restricted maximum likelihood (REML) approach for the examination of variances and calculation of parameter estimates. Analysis was done in JMP 8 (JMP 1989–2007), and P values for significance tests of differences between the two population types were adjusted using sequential Bonferroni correction (Holm 1979).

On the basis of allele frequencies at seven nuclear microsatellite loci, we calculated selfing in offspring per maternal tree and over entire populations as $s = 1 - t_m$, using MLTR 3.2 (Ritland 2002) and running 1,000 bootstraps to infer standard deviations. Levels of inbreeding in adult trees

were calculated as $F_{IS} = 1 - (H_o/H_e)$ (where H_o and H_e are observed and expected heterozygosity, respectively; Wright 1951) for independent samples of mature *P. cembra* trees from the seven study populations (16 individuals per stand; Gugerli et al. unpubl. data) using FSTAT 2.9.3. Finally, population-wise fitness values were correlated to selfing in offspring using Pearson's *r* (Wessa 2012).

Results

Parental seed production and offspring performance

The proportion values of filled seeds among fully developed seeds, as an inverse measure of embryo abortion used to adjust the FV germination success, showed differences between population types (Fig. 2). In the core populations, 67.52 % ± 0.03 SE of the fully developed seeds that we had fractured contained viable embryos. In contrast, 23.06 % ± 0.03 SE of the fully developed seeds collected in the peripheral populations were filled with embryos, reaching a minimum of 3.93 % ± 0.04 SE in population FL.

Differences in fitness values between the two population types varied among the four levels examined in that only parental seed production and germination success showed a significant effect (Fig. 3, Table 3). For parental seed production, the peripheral populations showed significantly lower FVs than the core populations (Table 4, Fig. 3a). Looking separately at all three RPCs that contributed to parental seed production showed that the differences between population types were large for each of these
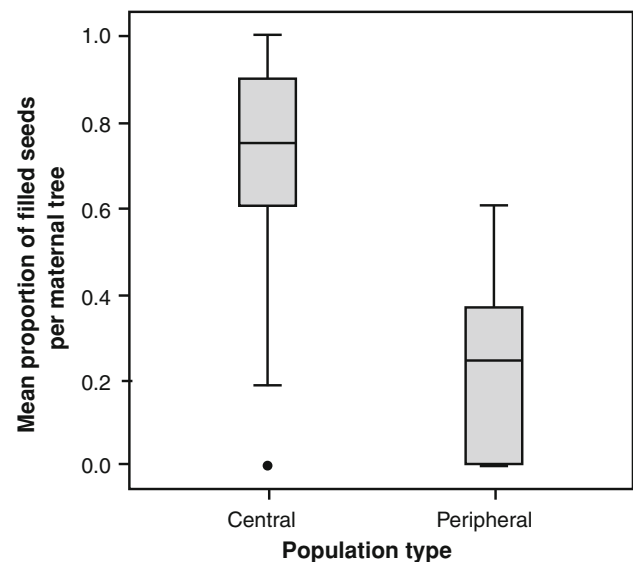
Fig. 2 *Boxplots* showing the mean proportion of filled among fully developed seeds, calculated for maternal trees from core and peripheral populations of *Pinus cembra* in the eastern Swiss Alps

factors (Table 3). Average total seed set was two times higher in core than in peripheral populations. On average, almost 92 % of seeds were fully developed in core populations, meaning that about 8 % of the total seed set had been aborted during the first year of seed development. In contrast, almost 44 % of ovules had been aborted in peripheral populations. Likewise, the weight of fully developed seeds averaged more than 220 mg in core populations, while the mean seed weight in peripheral populations was less than 170 mg.

Regarding seedling performance, germination success was significantly lower in peripheral than in core populations (Table 4, Fig. 3b). On average, only 10.09 % ± 0.02 SE of the filled seeds from the peripheral populations germinated, contrasting a germination success of 50.88 % ± 0.02 SE in filled seeds from the core stands. The lowest value was observed in the peripheral population FL, where no seedling emerged. Furthermore, the influence of the

covariate average seed weight in the GLM on germination success was significant, together with the significant interaction between seed weight and population type (Table 4). No significant differences were detectable in seedling survival and first-year growth between population types (Table 4, Fig. 3c, d). The influence of seed weight was significant for both fitness parameters, but not its interaction with population type (Table 4).

Genetic analyses

We obtained multilocus genotypes of 651 embryos among 42 out of the 54 Swiss stone pine mother trees sampled in 7 populations from two different population types. In the peripheral populations, we obtained data for two of the three sampled populations only, because we found no more than two embryos among all seeds in stand FL (Table 1).
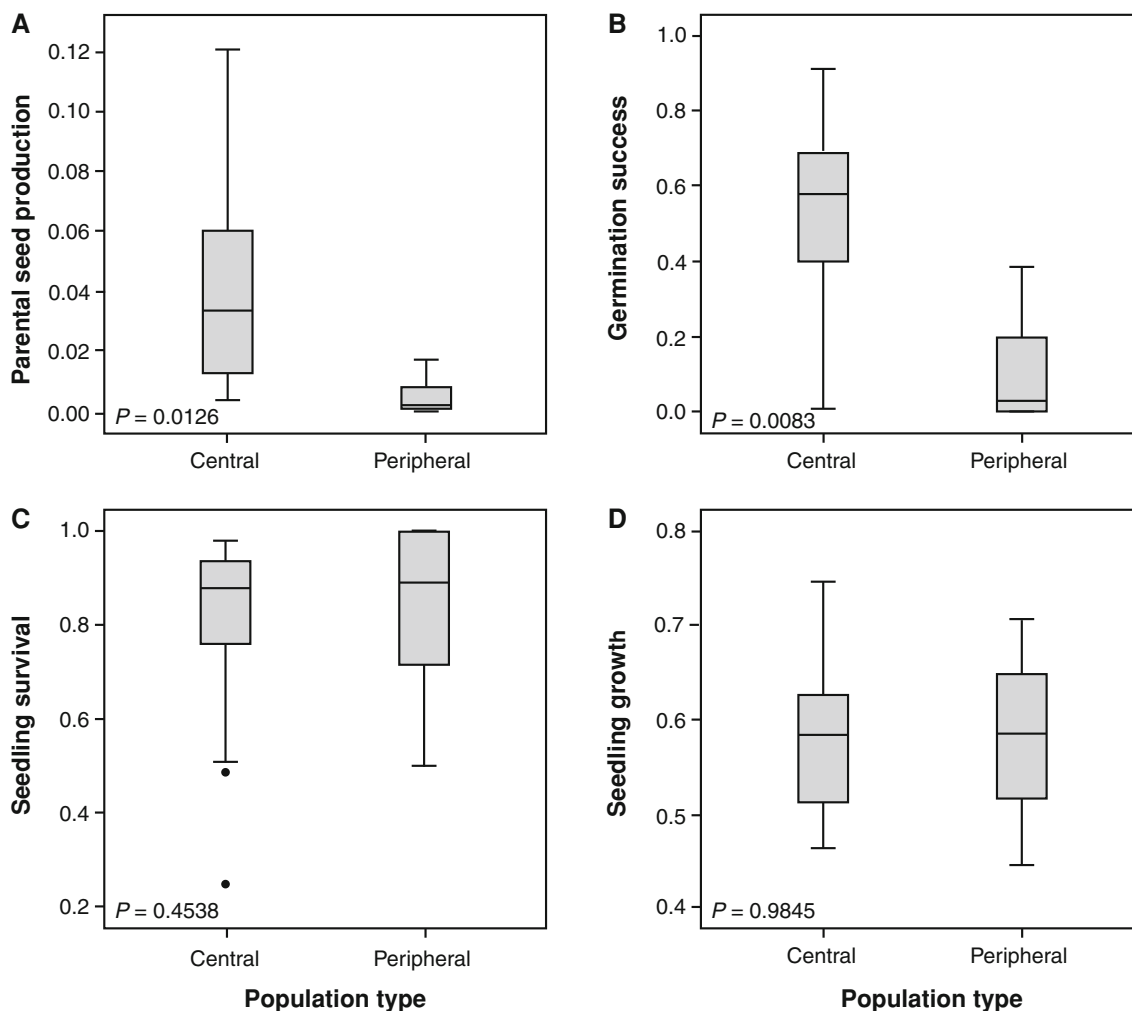
Fig. 3 Boxplots showing mean performance at four life stages, calculated for maternal trees from core and peripheral populations of Pinus cembra in the eastern Swiss Alps. a Parental seed production, b germination success, c seedling survival and d seedling first-year growth. P values refer to general linear models (see "Materials and methods" section)

Table 3 Seed production traits estimated for cones collected from *Pinus cembra* from core and peripheral populations in the eastern Swiss Alps

Population	Total number of seeds	Percent of aborted ovules	Proportion of filled seeds	Number of fully developed seeds	Average weight per fully developed seed (g)
Core	55.9 (30.1)	8.53 % (14.29)	0.71 (0.22)	51.1 (29.4)	0.225 (0.074)
Zernez	79.5 (29.6)	8.77 % (13.81)	0.39 (0.27)	72.8 (30.3)	0.162 (0.048)
St Moritz	37.5 (19.9)	14.09 % (17.98)	0.73 (0.13)	31.1 (16.2)	0.245 (0.076)
Avers	60.5 (22.1)	1.22 % (2.35)	0.86 (0.11)	59.8 (22.0)	0.261 (0.055)
Davos	38.5 (18.0)	7.59 % (1.32)	0.86 (0.12)	35.1 (17.5)	0.268 (0.049)
Peripheral	27.1 (21.1)	43.71 % (37.05)	0.21 (0.16)	13.0 (14.7)	0.167 (0.065)
Rautialp	24.6 (14.1)	34.81 % (36.26)	0.29 (0.21)	16.2 (15.4)	0.179 (0.070)
Neuenalp	45.0 (24.6)	67.72 % (26.43)	0.32 (0.23)	13.6 (15.0)	0.147 (0.062)
Flumserberge	3.6 (2.9)	26.38 % (35.76)	0.03 (0.05)	2.4 (1.9)	0.164 (0.033)

Values per cone are averaged across maternal trees to obtain population mean, with standard deviations in parentheses. Total number of seeds and average weight per fully developed seed both include filled and empty seeds (see "Materials and methods")

Table 4 Results of mixed hierarchical general linear models on parental seed production and early life stage fitness in *Pinus cembra* offspring from core and peripheral populations in the eastern Swiss Alps

Source of variation	F	P	Pearson's r (fitness—selfing)		
			Core	Periphery	Overall
Parental seed production			0.212	0.250	0.079
Population type	13.22	0.0126*			
Germination success			0.081	0.450	0.008
Population type	17.15	0.0083*			
Covariate seed weight	12.24	0.0005*			
Seed weight × population type	6.29	0.0125*			
Seedling survival			0.131	−0.248	0.003
Population type	0.63	0.4538			
Covariate seed weight	11.71	0.0007*			
Seed weight × population type	0.25	0.6157			
Seedling first-year growth			−0.056	−0.300	−0.135
Population type	0.00	0.9845			
Covariate seed weight	13.38	0.0004*			
Seed weight × population type	0.15	0.6947			

Pearson's correlations between each fitness value and estimated selfing rates, calculated at the level of maternal trees, are given for core, peripheral, and all populations

F values and P values are given for population type, covariate and their interaction. All Pearson's correlation coefficients are non-significant at $\alpha = 5$ % after Bonferroni correction

At the population level, selfing rate estimates $s = 1 - t_m$ were >0 in seed families of all populations except AV (Table 1), on the basis that t_m values per population were significantly lower than 1 given the bootstrapped estimates of standard deviations. The highest selfing rate was estimated in the peripheral population NA (0.562), whereas all other values of s were low (Table 1). Fixation indices of adult trees were all not significantly different from 0 (Table 1), i.e. they did not indicate deviations from Hardy–Weinberg equilibrium.

Seedling performance and inbreeding coefficients

We failed to detect any significant relationship between inbreeding, estimated by selfing rate s, in seed families and means of fitness values per mother tree, be it over all populations or separately in core and peripheral populations, respectively (Table 4). Pearson's correlation coefficients between values of s versus both parental seed production and germination success revealed consistently positive r values. Seedling survival correlated positively in core populations,

but negatively in peripheral populations, yielding an overall correlation close to zero over all populations. Finally, seedling growth resulted in negative correlation with selfing.

Discussion

In this study, we combined fitness experiments using open-pollinated seed families sampled in large and small *P. cembra* populations with molecular genetic analyses, i.e. data on inbreeding estimates, from the same progenies. We found significant differences regarding parental seed production and germination success between the two population types, while offspring performance (survival and growth) did not differ. Additionally, we found striking differences in embryo abortion rates. We took these results as an indication of higher inbreeding depression at the earliest life stages in small and fragmented populations compared to large and contiguous populations, as predicted by theory. However, molecular markers did not reveal substantial inbreeding in the offspring, i.e. in developed embryos, since aborted embryos, potentially resulting from inbreeding, cannot be genotyped readily. Accordingly, we infer that inbreeding depression is expressed at the prezygotic and/or early embryonic phase, which is in part why we failed to detect a relationship between the selfing estimates in developed embryos and experimental fitness of their siblings as detailed below.

No relationship between offspring performance and inbreeding

Seedling performance did not correlate with potential inbreeding, as estimated by selfing as the most strict type of inbreeding, in our study on *P. cembra*. This finding is per se not surprising in light of the controversially discussed relationship between inbreeding, e.g.in terms of heterozygosity, and fitness (Savolainen and Hedrick 1995; Britten 1996; Slate and Pemberton 2002; Balloux et al. 2004; Pemberton 2004; Chapman et al. 2009; Hansson 2010). Moreover, it has to be considered that early acting inbreeding depression might have led to the underestimation of real inbreeding rates (Kärkkäinen and Savolainen 1993; Husband and Schemske 1996; Rajora et al. 2002; del Castillo and Trujillo 2008). Since conifers have one of the highest numbers of lethal equivalents known (Namkoong and Bishir 1987; Savolainen et al. 1992), an increase in homozygosity is likely to have strong negative effects on offspring. In line with this, inbreeding in conifers has been shown to cause high embryo abortion rates during seed development (Koski 1971; Charlesworth and Charlesworth 1987; Kärkkäinen et al. 1999). In our study, around 76 % of the fully developed seeds collected in the peripheral stands were empty, while seeds from large populations showed on average 30 %

embryo abortion. In accordance with other studies on out-crossed conifers (e.g. Mosseler et al. 2000; Rajora et al. 2002), we take these high embryo abortion rates as an indication of inbreeding depression at the earliest life stage. At the same time, our results underline that molecular genetic investigations on inbreeding in predominantly out-crossing species—even if derived at an early life stage, i.e. in embryos—will not necessarily elucidate the magnitude of inbreeding. This is because pre- or early post-zygotic abortion can hardly be assessed through molecular markers given the absence or minimal size of respective embryos. Hence, a significant relationship between inbreeding and offspring performance is difficult to detect, even more so as inbreeding coefficients based on neutral molecular markers may not be representative of fitness-relevant loci.

In accordance with the results from other studies on stone pines (Krutovskii et al. 1995; Politov and Krutovskii 2004; Politov et al. 2006, 2008; Bower and Aitken 2007), we found significantly positive estimates of selfing rates in part of the progeny arrays, but no signs of inbreeding in adult samples. A decrease in heterozygote deficiency with increasing age is considered as an indicator for selection against inbreds during successive life stages in trees (Morgante et al. 1993; Krutovskii et al. 1995; Mitton et al. 1997). However, one should note that genetic parameters based on genotypes derived from viable embryos likely underestimate true levels of inbreeding because the genotypes of aborted zygotes are neglected.

Lower parental seed production in peripheral populations

Peripheral populations performed significantly worse regarding parental seed production than did core populations. Seed production combines processes that take place over a 2-year period in *P. cembra* (Zoller 1991). Hence, the reproductive cycle is influenced by various abiotic and biotic factors, particularly climatic conditions (Houle and Filion 1993, and references therein) over an extended period. According to Schnidrig (1935), fruit set in *P. cembra* depends mainly on the temperature during cone initiation, which suggests that adverse weather conditions might have caused lower total seed numbers in the peripheral compared to the core populations. Moreover, masting cycles may result in low seed crop in a particular year. Furthermore, *P. cembra* trees in the peripheral stands often grow on rocky outcrops, since these offer safe sites from competition by abundant *Picea abies* and are also less prone to grazing. At the same time, soil characteristics along with resource limitation at these sites might cause lower seed weight in peripheral compared to core populations.

The third parameter examined regarding parental seed production was the rate of unfertilized ovules, which

depends on pollination success (Sarvas 1962). Pollen is wind-dispersed in pines and was shown to travel large distances (Koski 1970; Salzer 2011). Therefore, high pollen abundance could be assumed in stands of *P. cembra*. Nevertheless, our peripheral populations showed high rates of unfertilized ovules, indicating pollen limitation in addition to the high embryo abortion rate. In these small stands, the number of local compatible mates is likely reduced compared to populations with large census sizes. Together with a lower population density, one can assume that total pollen availability is lower and less even in peripheral than in core populations, which in turn increases the likelihood of correlated mating, biparental inbreeding or even selfing (Kamm et al. 2011). The elevated genetic differentiation among these peripheral as compared to core populations (Gugerli et al. 2009) also indicates that gene flow among fragmented stands, which potentially counteracts inbreeding, is limited.

Germination success, seedling survival and first-year growth

The peripheral populations showed significantly lower germination success compared to core populations, while seedling survival and growth did not differ between the two population types. This could partly be due to maternal effects, known to be important for seedling performance (Bishir and Namkoong 1987; Roach and Wulff 1987; Wolfe 1993; Lindgren and Wei 1994). In line with this, we detected a positive influence of seed weight on germination success, seedling survival and first-year growth, indicating that maternal resource allocation did affect offspring performance in *P. cembra*. Interestingly, the interaction between population type and seed weight was only significant for germination success, but not for seedling survival and growth. Thus, seed weight influenced seedling emergence to a different degree in core and peripheral populations. This observation might suggest that germination success does not necessarily increase, once a certain threshold value of seed weight is exceeded. Differences in germination success between the two population types were still significant after accounting for seed weight. Thus, other factors than maternal effects have to be considered as influencing germination in *P. cembra*. In conifers, inbreeding depression is thought to be a major cause for both embryo abortion and reduced performance in subsequent life stages, including germination, seedling survival and growth (e.g. Husband and Schemske 1996). While early life stages are expected to be largely influenced by the expression of highly deleterious alleles, moderately deleterious mutations are thought to have an impact on traits expressed at later life stages, such as vigour and metric traits (Mitchell-Olds and Guries 1986; Husband and Schemske 1996; Sorensen 2001). Thus, the similar performance of progeny from both population types

with increasing age could be explained by decreasing inbreeding effects. At the same time, sensitivity of inbreeding depression to environmental conditions has to be considered. Because inbreeding effects tend to be stronger in stressful environments (reviewed in Armbruster and Reed 2005), negative effects on inbred offspring performance in seedling survival and growth might be more pronounced under natural conditions than in the nursery. However, this could only be investigated by common garden experiments or reciprocal transplantation experiments under natural conditions, which were beyond the scope of this study.

Conclusions

The results of this study emphasize the importance of several factors determining reproductive output and potential for regeneration in *P. cembra*: a combination of lower pollen quantity and diversity, adverse weather conditions, resource limitation and higher rates of inbreeding are possible causes that have lead to reduced offspring performance in small and fragmented peripheral *P. cembra* populations compared to large and contiguous core populations in the central Alps. This limitation may hamper the long-term persistence of peripheral populations. The on-going changes in climatic conditions are predicted to result in a migrational shift of the species to higher altitudes (Casalegno et al. 2010). In *P. cembra*, migration almost exclusively depends on bird-mediated seed dispersal (Furrer 1955), in which context reproductive output and the proportion of viable seeds are crucial (Salzer 2011). Taken together, our observations suggest that small and fragmented *P. cembra* populations in the northern periphery of the Swiss Alps are potentially vulnerable owing to uncertain regeneration. Furthermore, our study clearly showed negative consequences of fragmentation and small population size on a long-lived and wind-pollinated tree species, which have formerly been considered to be less susceptible to such effects (Kramer et al. 2008).

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