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ORIGINAL ARTICLE

Spruce colonization at treeline: where do those seeds come from?

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At treeline, selection by harsh environmental conditions sets an upward limit to arboreal vegetation. Increasing temperatures and the decline of traditional animal raising have favoured an upward shift of treeline in the last decades. These circumstances create a unique opportunity to study the balance of the main forces (selection and gene flow) that drive tree migration. We conducted a parentage analysis sampling and genotyping with five microsatellite markers in all Norway spruce individuals (342 juveniles and 23 adults) found in a recently colonized treeline area (Paneveggio forest, Eastern Alps, Italy). Our goal was to evaluate local reproductive success versus gene flow from the outside. We were able to identify both parents among local adults for only 11.1% of the juveniles. In the gamete pool we sampled, two-thirds were not produced locally. Effective seed dispersal distance distribution was characterized by a peak far from the seed source (mean 344.66 m ± 191.02 s.d.). Reproductive success was skewed, with six local adults that generated almost two-thirds (62.4%) of juveniles with local parents. Our findings indicate that, although a few local adults seem to play an important role in the colonization process at treeline, large levels of gene flow from outside were maintained, suggesting that the potential advantages of local adults (such as local adaptation, proximity to the colonization area, phenological synchrony) did not prevent a large gamete immigration.

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Introduction

High mountain systems such as the Alps are particularly vulnerable to climate change (Theurillat and Guisan, 2001). The European Alps experienced a 2°C average increase in annual minimum temperatures during the last century, with a marked rise since the early 1980s (Beniston *et al.*, 1997). Change in land use is also evident for the alpine region, with a progressive abandonment of traditional cattle rearing during the last 150 years, and the consequent cessation of summer grazing at and above the treeline (Dirnböck *et al.*, 2003). These factors promote a progressive upward shift of the treeline ecotone (Kullman, 2002; Walther, 2003; Gehrig-Fasel *et al.*, 2007), the belt between the upper continuous forest limit and the upper altitude isolated trees (Körner 1998). In the Alps, the area formerly used for grazing has been slowly colonized by subalpine species such as Norway spruce, European larch and European stone pine (Stützer, 1999; Didier, 2001; Bolli *et al.*, 2007).

Upward migration is limited at treeline by selective pressure from unfavourable abiotic conditions on seedling establishment and growth. Low temperature severely limits tissue formation and seedling growth (Hellmers *et al.*, 1970; Körner, 1999). During winter, frozen soil strongly reduces water availability, whereas stems and shoots are exposed to water losses caused by high wind and intensive radiation (Baig and Tranquillini, 1980). Drought stress combined with frequent freeze–thaw events causes embolism damage in forest trees such as Norway spruce (Mayr *et al.*, 2003). Moreover, during the last decades, frost damage increased given the shorter snow cover, caused in the alpine region by reduced winter precipitation (Brunetti *et al.*, 2000). Harsh environmental conditions, increasing temperature and the marked decline of grazing make treeline colonization a unique opportunity to study the balance of the main forces (selection and gene flow) that drive tree migration.

Parentage assessed by genetic markers is a powerful tool to study pollen and seed dispersal and seedling establishment (Meagher and Thompson, 1987). Highly polymorphic markers and new statistical methods improved our ability to infer family relationships within natural populations (Marshall *et al.*, 1998; Gerber *et al.*, 2000; review by Jones and Ardren, 2003). For plant species, genetic markers primarily have been used to measure effective pollen dispersal and relative male fertility by paternity analysis. Despite the importance of pollination dynamics, fitness of adult plants depends on seedling establishment (Dow and Ashley, 1996). Therefore, the assessment of parentage of established seedlings is the only approach that allows documentation of genetically relevant dispersal events, the so called 'effective' dispersal of pollen and seeds (Cain *et al.*, 2000; Hardesty *et al.*, 2006). The resulting recruitment pattern is defined as the result of the interaction between dispersal and

survivorship functions. The theory was first proposed by Janzen (1970) and Connell (1971) ('J-C recruitment pattern model', and recently modelled by Nathan and Casagrandi (2004). In addition, parentage analysis seems to be a promising technique to assess the scale and the quality of long-distance dispersal (LDD) events (Wang and Smith, 2002; Bacles et al., 2006). The study of LDD is crucial for understanding how plants can respond to global environmental changes (Trakhtenbrot et al., 2005).

Here we focus on parentage analysis, based on microsatellite compatibility, applying a categorical allocation method (Jones and Ardren, 2003). The investigation of parentage at treeline can shed light on migration movements and reproductive success of adult trees in a colonization area characterized by extreme ecological conditions. Strong selective pressures could counterbalance the effects of high gene flow, acting on and filtering out the diversity of arriving propagules (Jump and Peñuelas, 2005). Colonization events in forest trees were modelled by Austerlitz et al. (2000). Their results show that, in species with a long juvenile phase and a delayed first reproductive event, the colonization process is primarily sustained by new migrants from the source population, in numbers large enough to mitigate founder effects. For several tree species, it was shown that founder effects did not occur after colonization events (Mariette et al., 1997; Raspé and Jacquemart, 1998; Cespedes et al., 2003; Lefevre et al., 2004), but along altitudinal gradients there could be a different trend. In fact, Taira et al. (1997) and Premoli (2003) found in Cryptomeria japonica and Nothofagus pumilio a decrease of within-population genetic variation with increasing elevation. On the contrary, in the only specific study we found in the literature, Truong et al. (2007), studying genetic differentiation (F_{st}) between a newly established population of Betula pubescens ssp. tortuosa above treeline and the continuous forest below, found high levels of gene flow and a low genetic structure in the newly established population at treeline. However, to our knowledge, contemporary patterns of gene flow specifically in a treeline area was never studied with high resolution markers.

This paper investigates the colonization process looking in particular for answers to earlier unanswered questions about the upward shift of treeline:

(1) where do established juveniles come from?
(2) do the few reproductive individuals grown and matured at treeline, being adapted to local environment and being closer to the colonization front, have a reproductive advantage in the colonization over the many individuals grown at lower altitude?
(3) what is the 'shape' of the effective seed dispersal distribution at treeline?

Materials and methods

Study species
Norway spruce (Picea abies (L.) Karst.) is one of the most common and important boreal and mountain timber species in Europe. Its natural range extends from France in the west (5° E longitude) to the Ural Mountains (55° E) in the east, and from northern Norway (70° N latitude) to Macedonia (42° N). It can be divided into a north-east European boreal distribution and disjunct areas in the central European mountains, as suggested by palinological and genetic data (Giesecke and Bennett, 2004). In Italy Norway spruce is naturally distributed in the Alps from Valle d'Aosta to Friuli–V.Giulia, with relic populations in the northern Appennines (Bernetti, 1995).

P. abies is a predominantly outcrossing species, with both pollen and seeds dispersed by wind (Burczyk et al., 2004). Sexual maturity is usually reached at the age of 20–70 years (Chalupa, 2007). It is a continental tree that tolerates high summer temperatures but initiates bud and shoot growth at relatively low temperatures (Skre, 1979), and it prefers moist soils with high seasonal water storage (Sutinen et al., 2002).

Study site and sample collection
The study area, located in the Paneveggio forest (latitude 46°18', longitude 11°45'), in the upper part of Valbona valley around and above Colbricon lakes, is included in the Paneveggio–Pale di S. Martino Natural Park (Trentino, Italy). The vegetation in the whole Valbona valley is a typical subalpine Norway spruce forest (Di Tommaso, 1983). The average annual temperature is 2.4 °C and the rainfall is 1316 mm per year at Passo Rolle, 3 km from the study site at the same altitude (Gandolfo and Sulli 1993). Snow cover persists for about 4–5 months in the forest at 1700 m a.s.l. (Cavada and Piussi, 1974).

The study area (200 × 800 m, 1927–2200 m a.s.l., Figure 1) is located across the treeline, from the timberline (the continuous higher boundary of closed tall forest) to the upper spruce individuals found, that represent the upper limit of the treeline ecotone. The continuous population begins below the lower limit of the plot, after a discontinuity of about 80 m where no adult trees were found. In the Forest Management Plan records of the Paneveggio Forest, available since 1878, the area was always classified as a pasture. It had been extensively used for cattle grazing until the early 1970s. At that time no spruce trees were present, except for few young individuals established in the lower part, around the Colbricon lakes (Bonardi personal communication; Motta and Nola, 1996). The history and the dynamics of the forest below the study area have been intensively studied (Motta & Nola, 1996; Motta et al., 1999, 2002; Motta, 2002, and references therein). In particular, a plot at 1865 m a.s.l. immediately below our study area was described by Motta et al. (1999) as 'located at the present upper limit of the pure spruce forest in an area used in the past for forest grazing' in the early 1990s. After the cessation of grazing, about 35 years ago, the study area was progressively colonized by Norway spruce, determining an upward shift of the spruce treeline ecotone. Spruce masting frequency in the Valbona valley was estimated in a long term investigation (1973–1992) between 1 and 3, with lower frequency at higher altitude (Mencuccini et al., 1995).

Needles were collected from all the 365 individuals (adults, seedlings and saplings) present in the colonization area. There was no evidence of logging in the area, that has been a strict reserve since 1992, and neither stumps nor dead trees were found. In summer 2004, after a mast year, individuals were classified as 'adults' (23) if strobili were present, otherwise they were considered 'juveniles' (342) (Figure 1). Every sampled individual was mapped, using a Leica DISTO3 laser distancemeter
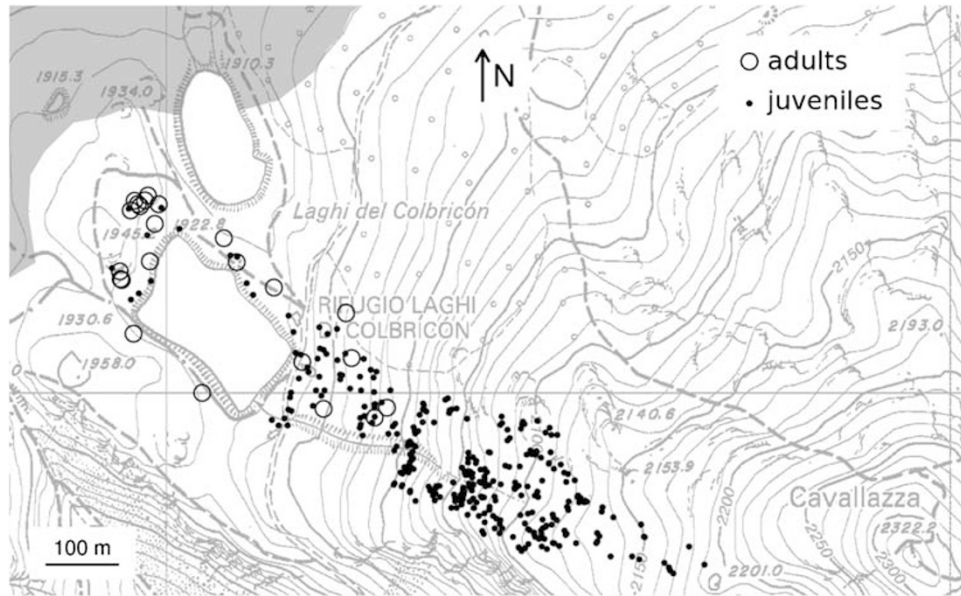
Figure 1 Map of the study plot. Open circles are adult trees and black circles are juveniles. The last black circle on the right side of the map is the upper sampled juvenile in the stand. The shaded area represents the continuous population below the study area, where the closest not sampled adults are located.
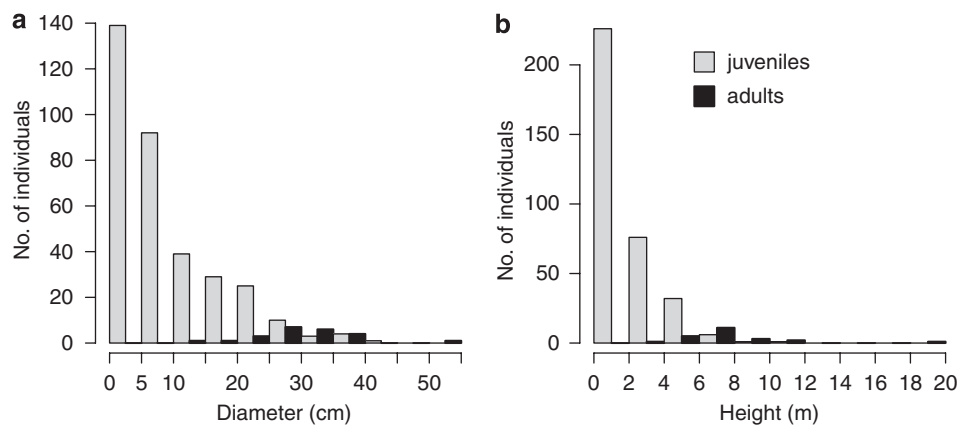

Figure 2 Distribution of diameter (**a**) and height (**b**) for adult trees (black bars) and juveniles (grey bars) in the study area. Mean basal diameter values: juveniles 9.38 ± 8.56 cm, adults 30.83 ± 7.98 cm. Mean height values: juveniles 1.96 ± 1.64 m, adults 7.76 ± 3.02 m.

and a compass, to determine its relative polar coordinates. Height and basal diameter of each individual were measured using a Haglöf Vertex III laser ipsometer and a measuring tape (Figure 2). Height and basal diameter were highly correlated ($r = 0.87$, $t_{363} = 34.09$, $P < 0.001$). Given the strict reserve nature of our study area, to investigate the relationship between age and size we took cores with Pressler drill from a subsample of 43 individuals among trees with basal diameter > 5 cm. A weak but significant linear relationship was found between age and height ($F_{1,41} = 6.02$, $P < 0.05$, $R^2 = 0.11$) and basal diameter ($F_{1,41} = 5.21$, $P < 0.05$, $R^2 = 0.09$).

DNA extraction and microsatellite analysis
Genomic DNA was extracted from about 70 mg of needle tissue, using Qiagen Plant DNeasy 96 isolation kit and Macherey–Nagel Nucleovac Vacuum Manifold. We analyzed three nuclear microsatellite marker loci developed by Scotti *et al.* (2002) and two developed by Pfeiffer

et al. (1997) (Table 1). Polymerase chain reactions (PCR) was carried out using a PTC100 thermal cycler (MJ Research, San Francisco, CA, USA) in a total volume of 25 µl. For SpAC03, EATC1B02, EATC2B02 and EATC1E03 primers reaction mix consists of 10 ng of template DNA, 100 µM of each dNTP, $10 \times$ Fermentas PCR buffer (100 mM Tris-HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% Nonidet P40), 2.5 mM MgCl₂, 0.1 µM forward primer and 0.25 µM reverse primer, 1 U Taq Polymerase (Fermentas, Glen Burnie, MD, USA). For SpAGG03 primer reaction mix consists of 10 ng of template DNA, 100 µM of each dNTP, $10 \times$ Fermentas PCR buffer (100 mM Tris-HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% Nonidet P40), 2.5 mM MgCl₂, 0.2 µM each primer and 1 U Taq Polymerase (Fermentas). PCR conditions were: 95 °C for 10 min, 7 touch-down cycles (95 °C for 30 s, Tₐ + 7 °C (−1 °C per cycle) for 30 s, 72 °C for 30 s), 28 amplification cycles (95 °C for 45 s, Tₐ for 45 s, 72 °C for 45 s) and a final extension step of 10 min at 72 °C for SpAC03, EATC1B02, EATC2B02 and EATC1E03 primers. PCR conditions for

Table 1 Description of the five nuclear SSRs used in this study

Locus	Length of PCR products	$T_a(°C)$	Numbers of alleles	H_e	EP_{sp}	EP_{pp}
SpAC03	133–157	53	11	0.773	0.395	0.765
SpAGG03	108–148	57	19	0.896	0.656	0.933
EATC1B02	192–252	58	12	0.403	0.086	0.376
EATC1E03	116–175	58	11	0.603	0.376	0.579
EATC2B02	173–243	55	20	0.651	0.267	0.674
Mean			14.6	0.655		
Overall					0.896	0.999

Abbreviation: SSR, simple-sequence repeat.
T_a is the annealing temperature, H_e is the expected heterozygosity, EP_{sp} and EP_{pp} are the single parent and parent pair exclusion probabilities. Overall values represent multilocus exclusion probabilities.

SpAGG03 primer were 94 °C for 10 min, 34 amplification cycles (94 °C for 45 s, T_a for 45 s, 72 °C for 45 s) and a final extension step of 10 min at 72 °C. Annealing temperatures are reported in Table 1

Data analysis

Parentage analysis was carried out using the software FaMoz (Gerber *et al.*, 2003). First, the variability and the resolution of our marker set were evaluated. Allele frequencies and expected heterozygosity (H_e) were calculated according to Weir and Cockerham (1984) using GENEPOP 3.4 (Raymond and Rousset, 1995). The consequent single parent and parent pair exclusion probabilities were calculated according to Jamieson and Taylor (1997) the formulae. Afterwards, parent-offspring relationships were investigated assigning LOD scores to every possible single parent and parent pairs in the stand (Gerber *et al.*, 2000), accounting for a mistyping error (*e*) of 0.01, as suggested by Bonin *et al.* (2004), and for the departure from Hardy–Weinberg equilibrium calculated from allelic frequencies. The thresholds for statistical significance of LOD scores were estimated after the simulation method described in Gerber *et al.* (2000). Using a categorical allocation approach (Jones and Ardren, 2003), we assigned parentage to parent pairs or single parents (when it was impossible to identify a couple) with the highest LOD score above the estimated thresholds. Cryptic gene flow, determined by false assignments to local parents of gametes sired by outside parents (Devlin and Ellstrand, 1990), was estimated using a simulation-based test in FaMoz. Simulations were run producing two offsprings of 1000 individuals from genotyped parents and from allele frequencies, and parentage was assigned using the above parameters and thresholds. In addition, confidence levels of estimated thresholds were assessed using CERVUS (Kalinowski *et al.*, 2007), running 10 simulations of parentage analysis on 10 000 simulated offspring.

Once parentage was resolved, we estimated the immigration rate from outside the plot at the genotype level as the number of genotypes entirely and partially produced outside, and at the gamete level as the ratio between gametes that originated from outside the plot and the total number of gametes sampled (Valbuena-Carabaña *et al.*, 2005). The proportion of juveniles with no identified parents and with only one identified parent represent, respectively, an estimate of seed and pollen gene flow from outside the study area (Bacles *et al.*, 2006).

Then, we used the assumptions postulated by Dow and Ashley (1996) to determine pollen and seed movements inside the sampling area. When only one parent matched a juvenile in the stand, this adult was considered to be the mother tree, whereas when a compatible parent pair was found, the closest parent was considered the mother tree whereas the farthest was considered the father tree. Although these assumptions seem particularly suitable in this context, being highly unlikely that many pollination events occurred from the colonization front to the forest below, followed by opposite and longer movements via seed toward the treeline area, they determine a conservative estimate of seed dispersal distance distribution, because of the possible mix-up between seed and pollen movements.

We used the dispersal data to build the effective propagule dispersal distributions. We called them 'effective' because dispersal distances were inferred from the positions of established individuals (Cain *et al.*, 2000). In particular, effective seed dispersal distribution represents the so-called recruitment pattern. On this latest distribution we fitted several families of curves (normal, lognormal, Weibull, Gamma and Clark's 2Dt) among the most suitable to describe the pattern found (Clark *et al.*, 1999; Jones *et al.*, 2005). As proposed by Jones *et al.* (2005), data regarding not assigned juveniles were treated as right censored data. These data were integrated in the estimate of the dispersal curve parameters, thus taking into account the probability, for not-assigned juveniles, to travel at least the minimum dispersal distance (MDD), defined as the distance from the location of the not-assigned juvenile itself to the low altitude border of the plot. Fitting based on a maximum likelihood approach was carried out using fitdistr function in R (R Development Core Team, 2005). Then we compared log likelihoods of the tested models, penalized by the number of parameters estimated, using Akaike's Information Criterion (AIC) calculated with AIC function in R. The lowest value of AIC represents the best fit (Akaike, 1992). We evaluated the relative support in the data of the best model calculating normalized Akaike weights (w_r) and the ratio of Akaike weights between the best model and the competing ones (ρ) (Hobbs and Hilborn, 2006).

The detection of effective seed dispersal distribution also allowed us to investigate LDD events taking place in the study area. Dispersal events can be classified as LDD if they exceed a case-specific biologically or physically relevant absolute threshold, or a relative threshold

inferred from the tail of the dispersal curve (for example, the ninety-ninth quantile) (Nathan, 2006). We assessed LDD by trying to quantitatively describe the tail of the dispersal curve obtained by parentage analysis. Furthermore, to quantify the effect of including MDD data into our fitting procedure and LDD estimation, we carried out maximum likelihood fitting only on dispersal data obtained from parentage assignments, after the method explained earlier. Once the best model was determined, we compared it with that obtained considering also MDDs.

Finally, we calculated individual reproductive success for every plant classified as an adult. Reproductive success was calculated as the total number gamete assigned to each adult (as maternal or paternal parent). The relationship between reproductive success and morphological traits (height and basal diameter) was evaluated using a generalized linear model with Poisson family link function (McCullagh and Nelder, 1989). Significance of the model was assessed with sequential analysis of deviance and its robustness was assessed by bootstrap (1000 resampling cycles on individuals). We used the glm, anova.glm and boot functions in R.

Results

A total of 73 different alleles were detected across the 365 sampled individuals. The number of alleles ranged from 11 to 20 for the five analyzed loci (mean 14.6). The expected heterozygosity for each locus ranged from 0.403 to 0.896. The high genetic variability characterizing our marker set warranted high single parent and parent pair exclusion probabilities (0.896 and 0.999, respectively). Table 1 summarizes the information about the five microsatellite loci used in the study.

As thresholds to determine statistical significance of parentage assignment we used the mean values (over 10 simulations) of the intersection between the two LOD scores distributions derived from a simulated offspring from inside the stand and a simulated offspring from outside the stand (2.792 ± 0.052 s.e. for single parent and 4.929 ± 0.047 s.e. for parent pair) (Gerber *et al.*, 2000). The chosen values guaranteed high confidence levels (single parent threshold: 90.1%; parent pair threshold: 78.0%), according to CERVUS simulation results. Using such thresholds, 38 juveniles out of the 342 sampled (11.1%) have a compatible parent pair inside the stand, 156 juveniles (45.6%) have only one compatible parent inside the stand and 148 juveniles (43.3%) have no compatible parents inside the sampling area (Figure 3). Among the 38 individuals with a compatible parent pair four were the products of self-pollination (the two parents detected were the same adult tree). At the gamete level, we found that 232 ($38 \times 2 + 156$) gametes were produced by a within-stand parent, whereas 452 did not match any adult, determining an immigration rate of 66.1%. The estimate of cryptic gene flow is slightly negative (-3.8%), interpretable as not different from 0 and thus confirming our gene flow estimation. We checked for potential assignment errors also comparing sizes of juveniles with one or two local parents (hereafter referred to as 'assigned juveniles') and most likely parent(s). In only 7 out of 194 cases (3.6%) assigned juveniles were taller and larger than their parents. Furthermore, to check for temporal trend in gene flow, we compared the
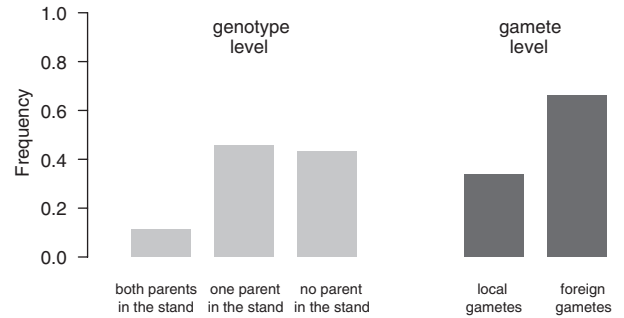

Figure 3 Gene flow at treeline. Proportion of juveniles with both parents, one parent, and no parent detected inside the sampling area (grey bars), and the proportion of locally and outside produced gametes (black bars).

distributions of diameter and height between assigned and not assigned juveniles. The two distributions were very similar both for diameter (Kolmogorov–Smirnov test: $D = 0.04$, $P = 1$) and height ($D = 0.07$, $P = 0.84$).

After Dow and Ashley (1996) assumptions, we were able to distinguish between pollination and dissemination events. Juveniles with one assigned parent are considered the result of gene flow via pollen from outside, whereas juveniles with no detected parents are the result of gene flow via seed from outside. We found that 45.6% of juveniles originated from external pollen and local mothers, whereas 43.3% arose from incoming seeds. On the other hand, considering both pollen and seed movements inside the study area, we detected only 38 pollination events (range 0–387.91; mean $143.67 \text{m} \pm 107.64$ s.d.), in comparison with 194 seed dispersal events. Thus only effective seed dispersal distances distribution was analyzed, whereas because of the small number of observation we were unable to accurately describe the distribution of pollination distances. The range of distances between mother trees and their offspring is 39.37–833.42 m (mean $344.66 \text{m} \pm 191.02$ s.d.) (Figure 4). Among the 194 seed movements detected 178 (91.7%) exceeded 100 m and 144 (74.2%) exceeded 200 m, 51 (23.3%) were longer than 500 m and two (1%) travelled over 800 m.

Among the curves tested to describe effective seed dispersal distribution, the Weibull curve best fitted our data, having the lowest AIC value and being largely more likely than other models. Only the Gamma curve was 1.5 times less likely, but the two models virtually exhibit the same behaviour (Table 2, Figure 4). The dispersal density function suggests that for a seed the probability of successful settling at long distance ($> 1 \text{Km}$) is high. The ninety-ninth percentile of the dispersal density function, often used as a threshold for defining LDD events, is placed at 1.8 Km (Table 3).

Repeating the fitting procedure analyzing dispersal data obtained from parentage assignment only (excluding MDDs), the Weibull was again the best model ($w_r = 0.998$). The comparison between the b parameters affecting the 'fatness' of the tail of the two Weibull curves (without MDDs: $b = 1.876 \pm 0.108$ s.e.; with MDDs: $b = 1.546 \pm 0.098$ s.e.), indicated that not including MDDs produces a significant thinner tail of the dispersal curve ($t_{340} = 3.371$, $P < 0.001$). Although the probability of reaching medium distances is similar at 1 Km between the two curves, at 2 Km the difference is much larger (~ 7
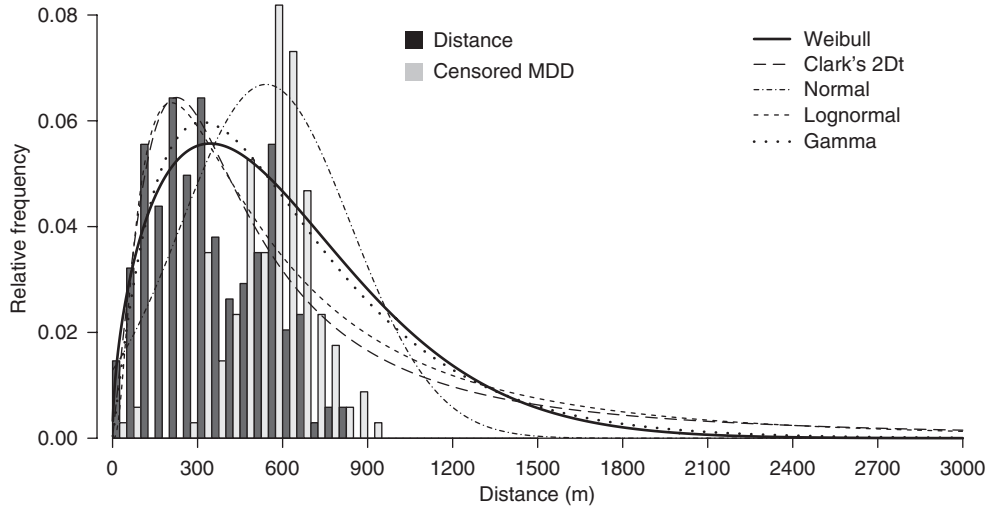
Figure 4 Distribution of inferred effective seed dispersal distances (black bars) and minimum dispersal distances (MDDs) for not assigned juveniles (grey bars) with the five fitted curves for estimated parameters. The Weibull curve (black solid line) best fits our data (Table 2).

Table 2 Parameter estimates and standard errors (s.e.) for the five fitted functions, sorted according to AIC values from the most to the less likely

Assumed dispersal function	Estimated parameters				AIC	w_r	ρ
	a	s.e.	b	s.e.			
Weibull	**674.991**	**33.117**	**1.546**	**0.098**	**2931.304**	**5.81×10^{-1}**	**1**
Gamma	3.255	0.302	0.009	0.001	2932.134	3.84×10^{-1}	1.51
Lognormal	6.253	0.060	0.958	0.052	2937.125	3.16×10^{-2}	18.36
2Dt	1.086×10^5	4.268×10^3	0.566	0.043	2941.568	3.43×10^{-3}	169.31
Normal	541.455	18.689	298.263	16.267	2979.173	2.34×10^{-11}	2.48×10^{10}

Abbreviation: AIC, Akaike's information criterion.
Parameters of the best fitting model are shown in bold type. a represents the ' scale' parameter and b the 'shape' parameter. w_r are normalized Akaike weights and ρ the ratio of Akaike weights between the best model and the competing ones.

Table 3 Comparison between best fitting models estimated with and without minimum dispersal distance (MDD) data

Distance (m)	Probability (without MDD)	Probability (with MDD)
800	2×10^{-4}	6×10^{-4}
1000	3×10^{-5}	4×10^{-4}
1200	3×10^{-6}	3×10^{-4}
1400	2×10^{-7}	2×10^{-4}
1600	1×10^{-8}	8×10^{-5}
1800	3×10^{-10}	4×10^{-5}
2000	8×10^{-12}	2×10^{-5}

Percentile	Distance (m) (Without MDD)	Distance (m) (With MDD)
80°	510	918
90°	607	1158
95°	698	1372
99°	878	1812

Abbreviation: LDD, long-distance dispersal.
Models were compared in terms of estimated probability for a seed to reach a given distance, and considering the position of key-quantiles in LDD events detection.

orders of magnitude), and taking into account MDDs the ninety-ninth percentile is about 1 Km further (Table 3).

Reproductive success was highly variable, ranging from 0 to 41 assigned gametes (mean 10.09 ± 8.80 s.d.). The most successful adult tree (#2336) sired 41 out of the

194 assigned juveniles (21.1%). The six most successful trees were the parents of almost two-thirds (62.4%) of the assigned juveniles. Considering all juveniles (assigned and not assigned), these six individuals were parents of 35.4% of the natural regeneration at treeline, and they produced 18.3% of all the sampled gametes (Figure 5). The reproductive success was significantly related with basal diameter (deviance = 19.325, $P < 0.001$) and not with height (deviance = 1.753, $P = 0.185$). By bootstrap, we found that the relationship between basal diameter and reproductive success was not robust (CI 95% of slope: -0.1195; 0.0362), and it was mainly sustained by one 'outlier' (tree #2336). In fact, no significant relationship was found if this individual is excluded from the model (basal diameter: deviance = 2.556, $P = 0.110$; height: deviance = 0.053, $P = 0.818$).

Discussion

Gene flow from outside is high in the study area at treeline. With our chosen parameters and thresholds, 66.1% of gametes originated from outside the stand, and only 38 out of the 342 investigated genotypes were compatible with parent pairs inside the treeline area. It is known that forest trees show extensive gene flow, especially when wind is the dispersal vector for pollen or seeds (Nathan *et al.*, 2002; Robledo-Arnuncio and Gil,
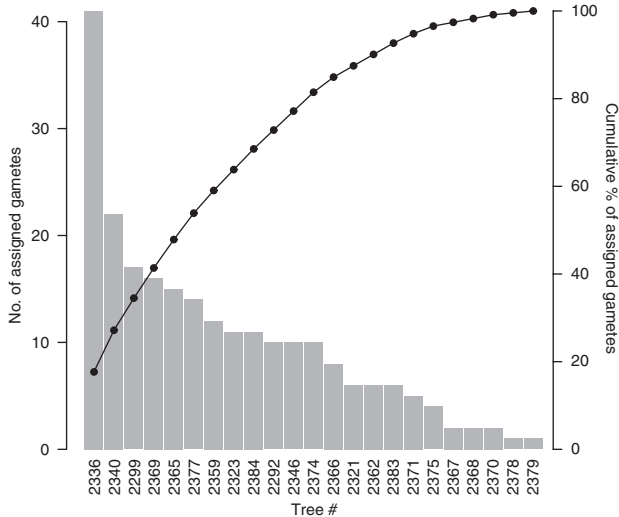
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Figure 5 Distribution and cumulative curve of reproductive success, measured as the number of assigned gametes per adult tree.

2005). In *Fraxinus excelsior*, a wind dispersed species with seeds one order of magnitude heavier than *P. abies*, Bacles *et al*. (2006) estimated seed immigration rate ranging from 50 to 70% in three remnants in an extended fragmented landscape (900 ha). Our results are also similar to those obtained in earlier studies on *P. abies*. Leonardi *et al*. (1996) found a weak spatial structure in a naturally regenerated uneven aged alpine stand, evidence of high gene flow. Scotti *et al*. (2008) in a mixed alpine stand found high gene flow via pollen and more spatially limited seed dispersal. Paternity analyses in *P. abies* seed orchards showed that pollen contamination is usually high (69–90%) and self-fertilization is low (0–6%) (Paule *et al*., 1993; Pakkanen *et al*., 2000; Burczyk *et al*., 2004). Such estimates are obtained before seed dispersal and seedling establishment. We found that 45.6% of juveniles originated from external pollen, but our estimate also encompasses events occurred after seedling establishment in a potentially selective environment such as treeline. However, to our knowledge, a direct estimate of effective gene flow by means of parentage analysis has never been carried out either in *P. abies* or at treeline.

In a dynamic environment such as the alpine treeline, the ratio between effective gene flow from outside and from inside is the result of a balance between reproductive, dispersal and establishment success of local versus lower-elevation adults. The contiguous population of trees located at lower elevation could have several advantages over local adults. First of all, it is numerically much larger with respect to the few adults located inside the treeline ecotone. Furthermore, trees located at lower elevation usually have relatively higher fecundity, produce seeds more frequently (Stevens and Fox, 1991) and of better quality (Oleksyn *et al*., 1998). Austerlitz *et al*. (2000) also pointed out that during forest tree colonization gene flow from the source population contributes more to recruitment than local reproduction, and the effect increases with the delay of the first reproductive event. Juveniles originated from lower elevation adults

could have an additional advantage due to increasing temperatures, because their parents are more adapted to temperatures now characterizing the treeline ecotone (Jump and Peñuelas, 2005). On the other hand, local adults have a significant proximity advantage and could be thought more adapted to the harsh treeline conditions. In a common garden experiment Oleksyn *et al*. (1998) showed how high altitude Norway spruce populations are effectively characterized by several potentially adaptive features (higher needle N%, light-saturated net photosynthesis and dark respiration rate, and a greater allocation to roots than to stem and needles). Furthermore, there are usually relevant differences in flowering phenology among trees at different altitudes that could hamper pollen immigration (Rathcke and Lacey, 1985). In a recent study carried out at treeline analyzing genetic differentiation, Truong *et al*. (2007) detected a large number of migrants per generation ($N_m = 48$) between a newly established *B. pubescens* population above treeline and two populations in the forest below. Our results show how apparently large levels of gamete immigration from outside were maintained at treeline suggesting that the potential advantages of local adults were not sufficient to overcome gamete immigration from the forest below.

Reproductive success of adults at treeline is highly skewed. Our six most successful adult trees are gamete donors for about a third of all the juveniles in the sampling area. Despite the large amount of gene flow from outside, a few among the 23 adults produced a disproportionate part of the natural regeneration, especially considering the large number of spruces living below treeline and the well-known recruitment limitations at treeline (Stevens and Fox, 1991; Oleksyn *et al*., 1998). Similar uneven reproductive success has been reported in many studies based on parentage analysis on herbaceous and tree species (Meagher and Thompson, 1987; Dow and Ashley, 1996; Aldrich and Hamrick, 1998; Schnabel *et al*., 1998; Sezen *et al*., 2005). We found that reproductive success is not related to tree size, and that tree size is weakly related with age. Hence we found no clear evidence that reproductive dominant trees had the advantage to be the first colonizers, contributing to more reproductive events simply because they had more time and space to spread their propagules.

The estimated average and maximum effective seed dispersal distances, 344.66 m and 833.42 m, respectively, are high in comparison with what found in other parentage studies on forest trees. In two studies carried out on the wind-dispersed conifer *P. pinaster*, Gonzalez-Martinez *et al*. (2002, 2006) found mean seed dispersal distances ranging from 26.53 m to 58.16 m, but *P. pinaster* has heavier seeds that limit its dispersion and their studied area was smaller. In three oak species mean seed dispersal distances detected by means of parentage analysis ranged between 14 and 42 m, but in these species dispersal is mainly determined by gravity, and the rare LDD events are caused by birds and rodents (Dow and Ashley, 1996; Valbuena-Carabaña *et al*., 2005). Our results can be explained considering the low mass (~ 10 mg) of *P. abies* seeds and their seed wing (Bernetti, 1995). In a parentage study conducted analyzing maternal tissues of *Jacaranda copaia* seeds, a common neotropical wind-dispersed tree with seed mass comparable with *P. abies*, a value similar to our maximum seed

dispersal distance was found (Jones *et al.*, 2005). This suggests the importance of seed morphology in determining LDD potentiality in anemochorous species.

The detection of rare LDD events, which have historically been considered unimportant, is of primary importance in understanding species responses to environmental changes (Trakhtenbrot *et al.*, 2005, Nathan, 2006). Genetic methods seem particularly appropriate to provide more accurate LDD data (Alsos *et al.* 2007), although studies based on parentage analysis that can provide LDD estimates at the population level, are still scarce (Nathan, 2006). By means of parentage analysis on established juveniles, Troupin (2005) found in *Pinus halepensis* colonization events over 500 m long, and Bacles *et al.* (2006) recorded individuals established up to 1.4 Km from their mother tree in a fragmented population of *F. excelsior*. Long-distance dispersal and establishment events were also detected in other tree species by Goto *et al.* (2006) and Hardesty *et al.* (2006) using the same approach. We found that the probability of reaching distances as long as 2 Km is still high, especially considering the large reproductive output of conifers, and how taking into account MDDs provides a better estimation of the tail of the curve, reducing underestimation errors. Our results confirm the high potential of Norway spruce seeds for LDD, even if the location of adults in our plot (all on the lower side) and the greater available space in the upper part of the colonization area certainly contributed to relatively high-dispersal distances.

Detected effective seed dispersal distances have an unimodal distribution with a peak several hundreds of meters away from the seed source. Most of the studies on dispersal in anemochorous trees were actually based on seed dispersal (Greene *et al.*, 2004; Clark *et al.*, 2005), whereas the shape of our distribution, being based on established juveniles, reflects both the dispersal capability of *P. abies* at treeline and the effect of post-dispersal events influencing seed and juvenile survivorship (Wang and Smith, 2002). Our results could be interpreted by hypothesizing an increasing survivorship coupled with the well-known decreasing seed dispersal probability from the seed source, as in Janzen–Connell recruitment pattern. J–C effects had been hypothesised in estimating effective seed dispersal distribution using parentage analysis in *P. halepensis* (Nathan *et al.*, 2000; Troupin, 2005), *P. pinaster* (Gonzalez-Martinez *et al.*, 2006) and *Simarouba amara* (Hardesty *et al.*, 2006). However, J–C pattern implies that the increasing survivorship curve depends on the density of predators or pathogens. Although seed predation is not the main dispersal vector in *P. abies*, spruce seeds are harvested by insects, small birds and rodents. Moreover, in the lower part of the study area the ground has a patchy rhododendron cover. Norway spruce could be parasitized by *Chrysomyxa rhododendri*, a needle rust with a host shift between *Rhododendron* sp. and *P. abies* that determines a degradation of the photosynthetic apparatus in infected needles (Bauer *et al.*, 2000, Mayr *et al.*, 2001). It is possible that similar infections might determine a higher survival far from adult trees because of a lower-host density. Other biotic and abiotic factors (such as light and competition for other resources) could favour locations far from parents. The particular spatial distribution of adults and juveniles in our study area might also contribute to the observed effective seed dispersal distribution, because most of the juveniles are located in the upper part of the plot, but this is a normal condition studying tree colonization. In addition, the presence of a small lake in the lower part of the study area might have reduced the frequency of short-distance dispersal events, but not long-distance dispersal estimates. However, the small lake covers only 10% of the study area, and seed dispersal distances referred only to juveniles assigned to adults above the small lake showed again a unimodal distribution with a peak far from the seed source (results not shown).

In conclusion, parentage analysis provided valuable hints about the origin of juveniles involved in treeline colonization. Gene flow from outside was high, even though our results show that some local adults had remarkable reproductive success and played a critical role in the colonization process. The large gamete immigration and the high percentage of trees established far from their mother trees confirm and quantify the high-dispersal capability of *P. abies*. Further research based on alternative approaches, such as reciprocal transplant experiments, ecophysiological measurements, seed-set evaluation and phenology assessment, is needed to assess the degree of local adaptation and to refine our knowledge of dispersal processes at treeline. Also the pattern of gamete immigration from the forest below can be clarified by increasing the sampling effort to determine where the unassigned gametes come from and the relative contribution of trees at different altitudes to treeline colonization. However, parentage investigation proved to be a reliable starting point in studying the complex ecological issues of treeline upward shifting.

Acknowledgements

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