

No effect of elevation and fragmentation on genetic diversity and structure in *Polylepis australis* trees from central Argentina

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Abstract Phenological differences in flowering arising along elevational gradients may be caused by either local adaptation or phenotypic plasticity. Local adaptation can lead to reproductive isolation of populations at different elevational zones and thus produce elevational genetic structuring, while phenotypic plasticity does not produce elevational genetic structuring. In this study, we examined the effects of elevation and fragmentation on genetic diversity and structure of *Polylepis australis* populations, where individuals exhibit phenological differences in flowering along an elevational gradient. We assessed the polymorphism of amplified fragment length polymorphism markers in adults and saplings from one conserved and one fragmented forest covering elevations from 1600 to 2600 m asl. Over 98% of variation was found within populations, and we found very low and similar genetic differentiation along elevational gradients for adults and saplings in both continuous and fragmented forests. In addition, there was no significant relationship between genetic diversity and elevation. Results indicated that phenological differences along elevational gradients are more likely caused by phenotypic plasticity than local adaptation, and fragmentation does not appear to have affected genetic diversity and differentiation in the studied populations. Results therefore imply that if necessary, seeds for reforestation purposes may be collected from different elevations to the seeding or planting sites.

Key words: amplified fragment length polymorphism, central Argentina, elevational gradients, fragmentation, *Polylepis australis*.

INTRODUCTION

Plant species that occur along wide elevational gradients may exhibit high phenotypic variation (Haider *et al.* 2012) as well as genetic differentiation among populations (Ohsawa & Ide 2008 in review; Shi *et al.* 2011). On the one hand, divergent selection pressures promote the evolution of traits adapted to the local environments, that is, local adaptation (Frei *et al.* 2014), which could lead to reproductive isolation at elevational belts and produce elevational genetic structuring. However, gene flow may counteract local adaptation through the constant influx of maladapted genotypes, a situation under which only strong selection pressure can lead to local adaptation (Lenormand 2002; Frei *et al.* 2014). On the other hand, phenotypic variation may also be due to phenotypic plasticity, that is, the ability of a genotype to adjust its phenotype to different environments

without genetic change (Ghalambor *et al.* 2007). Concerning within-population genetic diversity along elevational gradients, four observed patterns were derived in the review of Ohsawa and Ide (2008): (i) diversity peaks on higher slopes are attributed to various reasons like decreased human disturbance and/or historical down slope range shifts due to climate change, and adaptation (e.g. Gamperle & Schneller 2002), (ii) greater diversity at lower elevations is considered a result of bottlenecks occurring throughout upward range expansion (e.g. Quiroga *et al.* 2002), (iii) higher diversity at intermediate elevations is thought to result from optimal mid-elevation habitat conditions following the central-marginal hypotheses (e.g. Oyama *et al.* 1993), and (iv) constant values of genetic variability all over the gradients are attributed to extensive gene flow (e.g. Truong *et al.* 2007).

Fragmentation could also affect genetic diversity and differentiation within and among plant populations (Kramer *et al.* 2008). Theoretical predictions of reduced genetic diversity and elevated inbreeding following habitat fragmentation (Young *et al.* 1996) are

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upheld for a number of wind-pollinated temperate tree species (Sork *et al.* 2002; Jump & Penuelas 2006). However, some recent reviews emphasize that plant populations may be resilient to the effects of habitat fragmentation due to high levels of existing genetic diversity within populations and evidence that long-distance dispersal of pollen, even in fragmented habitats, is common in many tree species (Hamrick 2004; Kramer *et al.* 2008). In addition, the lack of the predicted decrease in genetic diversity found in some studies may simply be due to the long generation time of trees relative to the timing of fragmentation, so that not enough time has passed for drift to reduce genetic diversity (Hamrick 2004; Kramer *et al.* 2008). It would provide important information for implementing conservation strategies to understand the underlying genetic basis under phenotypic variations and the effects of fragmentation on genetic diversity and differentiation of plant species.

High altitude forests dominated by species of the *Polylepis* genus (Rosaceae) are one of the most endangered mountain forest ecosystems of South America, where conservation and reforestation of the species are a priority (IUCN 2014). *Polylepis australis* Bitt. is the southernmost species of the genus and it is endemic to Argentina, where it is distributed across a broad elevational range from 1000 m to 3000 m asl (Kessler & Schmidt-Lebuhn 2006). Its distribution has been substantially reduced in the last centuries due to fires and livestock browsing, with most forests being restricted to steep ravines and rocky outcrops (Cingolani *et al.* 2008). Remnants of *P. australis* have been exposed to differing levels of historical human impact, which allows for the comparison of continuous and fragmented forests (Cingolani *et al.* 2008; Renison *et al.* 2011). The historical and contemporary effects of forest fragmentation may therefore be assessed and compared by evaluating the different life stages of trees, with genetic variation among adults reflecting pre- or historical degradation effects, and more contemporary effects being observable among seedling or saplings (Hensen *et al.* 2012; Vranckx *et al.* 2012).

Previous studies provided useful information on patterns of genetic diversity in other *Polylepis* species in the Andes (Aragundi *et al.* 2011; Hensen *et al.* 2012; Quinteros-Casaverde *et al.* 2012; Gareca *et al.* 2013) including *P. australis* in Argentina (Julio *et al.* 2008, 2011; Hensen *et al.* 2011). Julio *et al.* (2008) developed genetic information for a population in a small degraded forest and a population in a large conserved forest using both RAPD and ISSR procedures. They reported that there was significant genetic divergence between the young and adult trees in the fragmented forest and between juveniles of both forests, but little genetic divergence was recorded in adult trees between fragmented and

continuous forests, suggesting possible diminished gene flow in recent times due to forest loss. In addition, using ISSR and AFLP markers respectively Julio *et al.* (2011) and Hensen *et al.* (2011) reported very little genetic divergence of older trees between different *P. australis* populations. No prior study has apparently examined the effects of elevation and human disturbance on genetic diversity and structure of *P. australis* populations, where individuals exhibit flowering dates ranging from August at the lowest elevations to November at the highest elevations (DR, personal observations). Here, we examined genetic diversity and differentiation among populations of *P. australis* in central Argentina, by detecting AFLP variation in adults and saplings from continuous and fragmented forests situated within elevational gradients ranging from 1600 to 2600 m asl. In this study, we originally hypothesized that: (i) With respect to *P. australis* adults, populations are genetically structured along elevational clines as a result of differences in flowering phenology related to elevation; and (ii) Lower genetic diversity and higher genetic differentiation should be found in saplings as compared to adults in fragmented forests but not in continuous forest.

METHODS

Species description

Polylepis australis is an evergreen tree (Rosaceae) that grows up to 14 m tall (Renison *et al.* 2011). While the species is diploid in its northern range, we carried out our study in its southern range, where the species is mainly tetraploid, with some populations having individuals with polyploidy levels of 2, 3 and 6 at low frequencies (Kessler *et al.* 2014). It is likely that *P. australis* is primarily an out-croser, as selfing could be hampered by protogyny (Seltmann *et al.* 2007, 2009). The lifespan of *Polylepis* species can reach up to hundreds of years (Solíz *et al.* 2009). In addition, the species has high pollen viability and longevity, and effective pollen flow (up to 80 km, Seltmann *et al.* 2009). Fruits are single-seeded nutlets, produced annually and dispersed by wind/gravity up to 6 m (Torres *et al.* 2008; Zimmermann *et al.* 2009).

Study area

The Córdoba Mountain range of central Argentina is characterized by a relatively wet, warm season during summer (October to April) and a dry, cold season during winter (May to September). We collected adults (>400 cm high) and saplings (<20 cm high) of 12 populations distributed every 200 m asl along two elevational transects with different disturbance histories covering 1600–2600 m asl (Fig. 1). One transect, leading to Mount Champaquí, followed the 'Los Tabaquillos' river through a relatively well

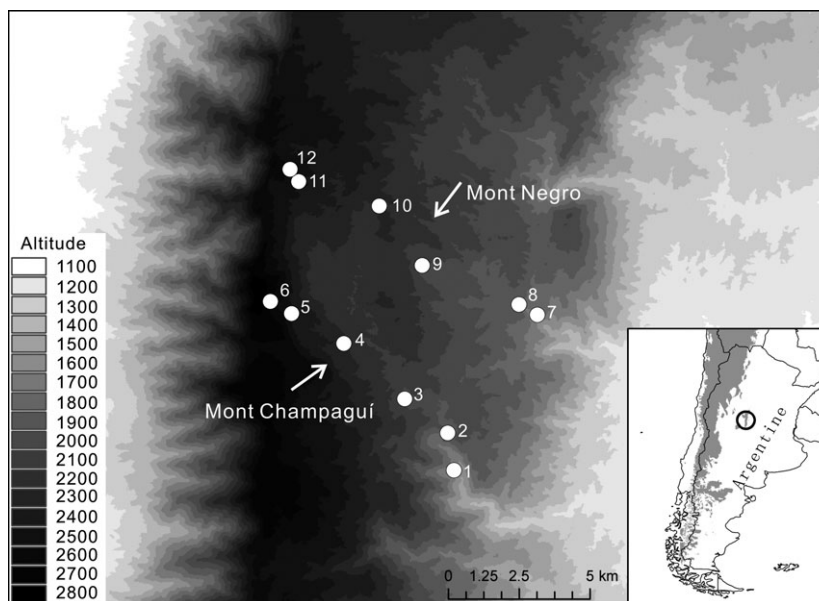


Fig. 1. The distribution of *Polylepis* in one continuous forest – Mont Champaquí (populations 1–6), and one fragmented forest – Mont Negro (populations 7–12).

preserved forest with a length of 22 km and a width varying from 0.2 to 1.2 km (hereafter the ‘continuous forest’). Here, *P. australis* forest represented around 40% of the landscape and was situated mostly within steep gorges formed by the river and intermingled with a mosaic of rock outcrops and grassland. This area represents one of the most extensive forests fragments found in central Argentina (Cingolani *et al.* 2004). The second transect, leading to Mount Negro, crossed a contiguous yet contrasting site across flatter and higher topographies, which also consisted of rock outcrops and grassland. In the latter transect, *Polylepis* forests had been extensively burned to support livestock rearing, trees were cut for use as fuel and for construction, and only isolated individuals or small forest patches of up to 50 trees could be found, typically within small inaccessible ridges or stream banks (hereafter the ‘fragmented forest’). This transect includes one of the most degraded areas of the Central Argentinean mountains (Cingolani *et al.* 2004), which has remained as such since at least the 1920s (M. Dominguez, personal communication). The geographical distances from the conserved forest to the fragmented forest range between 5 and 12 km. The minimum and maximum distances between the sampled populations were 0.76 km and 8.11 km in the continuous forest, and 0.5 km and 8.3 km in the fragmented forest respectively.

Sampling

Leaf samples were collected from a total of 144 adults and 144 saplings (height <20 cm) from six plots per transect with each plot measuring approximately 100 × 600 m. Given the size of adults and known growth rate and form analyses, adults were estimated to be older than 100 years (Suarez *et al.* 2008) while saplings were assumed to be <15 years old (Renison *et al.* 2015). Within each plot, we randomly sampled 12 adults and 12 saplings with leaves

being stored in bags with silica-gel prior to analysis. To minimize the chance of sampling closely related or genetically identical individuals, sampled trees were separated by at least 20 m and care was taken to ensure that plot sizes and distances between sampled trees were similar between the large forest and small forest transects.

DNA extraction and AFLP analysis

Genomic DNA was extracted from approximately 20 mg of silica-dried material using an ATMAB method (Doyle & Doyle 1987). The AFLP method followed Peng *et al.* (2015). We chose four primer pairs that amplified reliably and showed some degree of polymorphism in pre-tests: *EcoRI* AAG – *MseI* CAT, *EcoRI* AGC – *MseI* CAT, *EcoRI* AAC – *MseI* CAA and *EcoRI* ACT – *MseI* CAA. Reliable AFLP bands were recovered following the method of Ley and Hardy (2013). We generated an output file for automatic scoring on the Fragment Profile of the MegaBACE (Applied Biosystems, Foster City, CA, USA) package, which converted peak data into a binary allelic matrix. The output file was prepared for SPAGeDi v1.4 (Hardy & Vekemans 2002) to test for the reproducibility of peaks using broad sense heritability (H^2) and its significance, calculated as F_{ST} of Weir and Cockerham (1984). Peaks with $H^2 > 0.25$ and $P < 0.05$ were considered heritable, and we replicated more than 20% of the total individuals sampled for each primer. The AFLPdat R package (Ehrlich 2006) was used to transfer data between the different software packages used.

Data analysis

Given the polyploid nature of our study species, it was not possible to unambiguously estimate allele frequencies. In

accordance with Bonin *et al.* (2007), we analysed AFLP data employing both the band-based and fragment frequency-based approaches. We estimated the percentage of polymorphic bands (*PPB*) and Nei's expected heterozygosity (*He*) (Nei & Li 1979; Lynch & Milligan 1994) using a Bayesian method with uniform, prior distribution (Zhivotovskiy 1999) in the software AFLPSURV 1.0 (Vekemans *et al.* 2002). We assessed whether genetic diversity (*PPB* and *He*) differed significantly between adults and seedlings with paired *t*-tests using permutations in R 3.1.2 (R Core Team 2014, R-package 'broman', Broman & Broman 2014). The dataset was analysed assuming Hardy–Weinberg equilibrium. We analysed the effects of elevation on genetic diversity (*He* and *PPB*) for both adults and saplings using ANCOVA in R 3.1.2 (R Core Team 2014). Pairwise F_{ST} values (Weir & Cockerham 1984) were calculated for each population pair using Arlequin 3.5 (Excoffier & Lischer 2010). Significance was evaluated through 1000 permutations.

We employed a Principal Coordinate Analysis (PCoA) to distinguish similar genetic groups of individuals using the package 'vegan' (Oksanen *et al.* 2013) in R 3.1.2 (R Core Team 2014). We also used Analysis of Molecular Variance (AMOVA) performed with Arlequin 3.5 (Excoffier & Lischer 2010) to describe genetic structure and measure the amount of variation found within and among populations. *F* statistics and 1000 permutations were used to test significant levels of genetic structure.

Mantel tests and partial Mantel tests were performed to evaluate the linear correlation between genetic distance and elevational and geographic distance matrices. The Mantel test is simply a correlation between entries of two dissimilarity matrices, whereas partial Mantel tests use partial correlation conditioned on the third matrix (Legendre & Legendre 2012). Genetic distance matrices were constructed with F_{ST} values among populations. Mantel tests and partial Mantel tests were performed using the 'Vegan' package (Oksanen *et al.* 2013) in R 2.10.0 (R Core Team 2014). All tests were carried out with 1000 permutations, including Pearson assumptions considering rank order instead of real distance values to evaluate parametric relationships, with a limit of $P = 0.05$.

RESULTS

Genetic diversity

A total of 741 polymorphic markers were obtained from the four primer combinations (Appendix S1). Measures of within-plot diversity yielded high values (Table 1). Adults and saplings of *P. australis* did not differ in genetic diversity along elevations (ANCOVA: $F = 0.308$, $P = 0.58$), and genetic diversity did not correlate with elevation ($F = 0.061$, $P = 0.081$) (Table 1). Genetic diversity in the conserved forest ($PPB = 80.1$, $He = 0.265$ for adults; $PPB = 79.1$, $He = 0.265$ for saplings) was similar to that found in the fragmented forest ($PPB = 80.7$, $He = 0.264$ for

adults; $PPB = 78.6$, $He = 0.257$ for saplings). Adults and saplings showed no significant difference in genetic diversity in the conserved transect (PPB , paired permutation *t*-test: $P = 0.56$; He , paired permutation *t*-test: $P = 1$) or in the fragmented transect (PPB , paired permutation *t*-test: $P = 0.12$; He , paired permutation *t*-test: $P = 0.18$).

Genetic differentiation

For both adults and saplings, AMOVA indicated that 98% of the variation occurred within populations, while about 1% of the variation occurred among populations and 1% of the variation was partitioned among elevations (Table 2). Similar low levels of genetic differentiation were found between adults in the conserved forest ($F_{ST} = 0.017$) and in the fragmented forest ($F_{ST} = 0.012$). For saplings, genetic differentiation was absent in the continuous forest ($F_{ST} = 0.001$), while it was similar to that of adults in the fragmented forest ($F_{ST} = 0.018$). Low significant pairwise F_{ST} was detected among populations for both adults and saplings (Appendix S2). The PCoA revealed no clusters between the two forests or among the six elevational belts (Fig. 2) and there were no significant patterns of isolation by distance for the studied species in these regions (Table 3). Partial Mantel tests indicated that there was no significant isolation by elevation when considering geographic distance for either adults or saplings, although the Mantel test showed significant isolation by elevation for saplings (Table 3).

DISCUSSION

Low and similar levels of genetic differentiation along elevational gradients were found for adults and saplings. In addition, there was no significant relationship between genetic diversity and elevation. The result suggests that phenological differences along elevations may be caused by phenotypic plasticity and that fragmentation does not appear to have affected genetic diversity or differentiation in the studied populations.

Elevational gradients

Genetic diversity (*He* and *PPB*) reached similar levels across the elevational gradients and between degraded and conserved forests for both adults and saplings (Table 1). In accordance with a study of *Polylepis tarapacana* (Peng *et al.* 2015), the results highlight the lack of an elevational effect on genetic diversity as a result of an unrestricted gene flow

Table 1. Genetic diversity estimated as He and PPB for 12 populations of adults and saplings of *Polylepis australis* respectively

Pop	Lat (S)	Long (W)	Alt (m)	Adults			Saplings		
				n	PPB	He	n	PPB	He
Champaquí					80.1	0.265		79.1	0.265
Transect									
1	32.046	64.876	1600	11	84.2	0.275	12	78.9	0.257
2	32.034	64.877	1800	10	75.7	0.248	8	73	0.258
3	32.023	64.891	2000	8	76.6	0.268	10	81.8	0.276
4	32.006	64.91	2200	11	79.6	0.264	12	77.2	0.248
5	31.996	64.927	2400	11	82.7	0.264	10	85.4	0.296
6	31.992	64.934	2600	11	81.8	0.27	11	78.5	0.256
Negro Transect					80.7	0.264		78.6	0.257
7	31.997	64.849	1600	11	87.3	0.286	12	83.2	0.275
8	31.993	64.855	1800	11	79.3	0.254	11	79.7	0.262
9	31.981	64.886	2000	11	79.2	0.252	12	77.7	0.249
10	31.962	64.899	2200	11	80.1	0.267	12	78	0.251
11	31.954	64.925	2400	12	78.5	0.255	10	72.8	0.236
12	31.95	64.927	2600	12	80	0.27	12	80.4	0.271

N , the number of individuals; Lat, Latitude; Long, Longitude; Elev, Elevation.

Table 2. AMOVA for adults and saplings of *Polylepis australis* calculated from Arlequin ver. 3.5, partitioned by regions and elevations

Source of variation	d.f.	SS	VC	PV	F -statistics
Adults					
Among six elevations	5	634.7	0.512 Va	0.48	$F_{CT} = 0.005$
Among populations within elevations	6	694.4	1.045 Vb	0.99	$F_{SC} = 0.010^{**}$
Within populations	118	12327.1	104.47 Vc	98.53	$F_{ST} = 0.015^{***}$
Total	129	13656.2	106.03		
Among populations within Champaquí	5	621.7	2.0 Va	1.74	
Within populations	56	5887.9	105 Vb	98.26	$F_{ST} = 0.017^{***}$
Total	61	6509.6	107.0		
Among populations within Negro	5	592.9	1.3 Va	1.24	
Within populations	62	6439.2	103.9 Vb	98.76	$F_{ST} = 0.012^{***}$
Total	67	7032.2	105.2		
Saplings					
Among six elevations	5	607.5	0.49 Va	0.46	$F_{CT} = 0.005$
Among populations within elevations	6	664.3	0.55 Vb	0.52	$F_{SC} = 0.005$
Within populations	120	12560.7	104.67 Vc	99.01	$F_{ST} = 0.010^{***}$
Total	131	13832.6	105.71		
Among populations within Champaquí	5	537.2	0.1 Va	0.1	
Within populations	57	6060.1	106.3 Vb	99.9	$F_{ST} = 0.001^{***}$
Total	62	6597.3	106.4		
Among populations within Negro	5	626.8	2.0 Va	1.84	
Within populations	63	6500.6	103.2 Vb	98.16	$F_{ST} = 0.018^{***}$
Total	68	7127.4	105.1		

SV, Source of variation; SS, Sum of squares; VC, Variance components; PV, Percentage of variation. $***P < 0.001$.

among populations along this environmental gradient (e.g. Truong *et al.* 2007). We rejected seed dispersal as a mechanism maintaining *P. australis* polymorphism, as their wind-dispersed seeds mostly travel distances of <6 m (Torres *et al.* 2008; Zimmermann *et al.* 2009). However, a prior study of *Polylepis incana* showed decreasing genetic diversity with

increasing elevation (Table 3), which directly supports the hypotheses that this species has experienced genetic bottleneck due to upward range expansion (e.g. Quiroga *et al.* 2002).

For both forests, genetic differentiation among populations for both adults and saplings was not high enough to assume any genetic structuring of

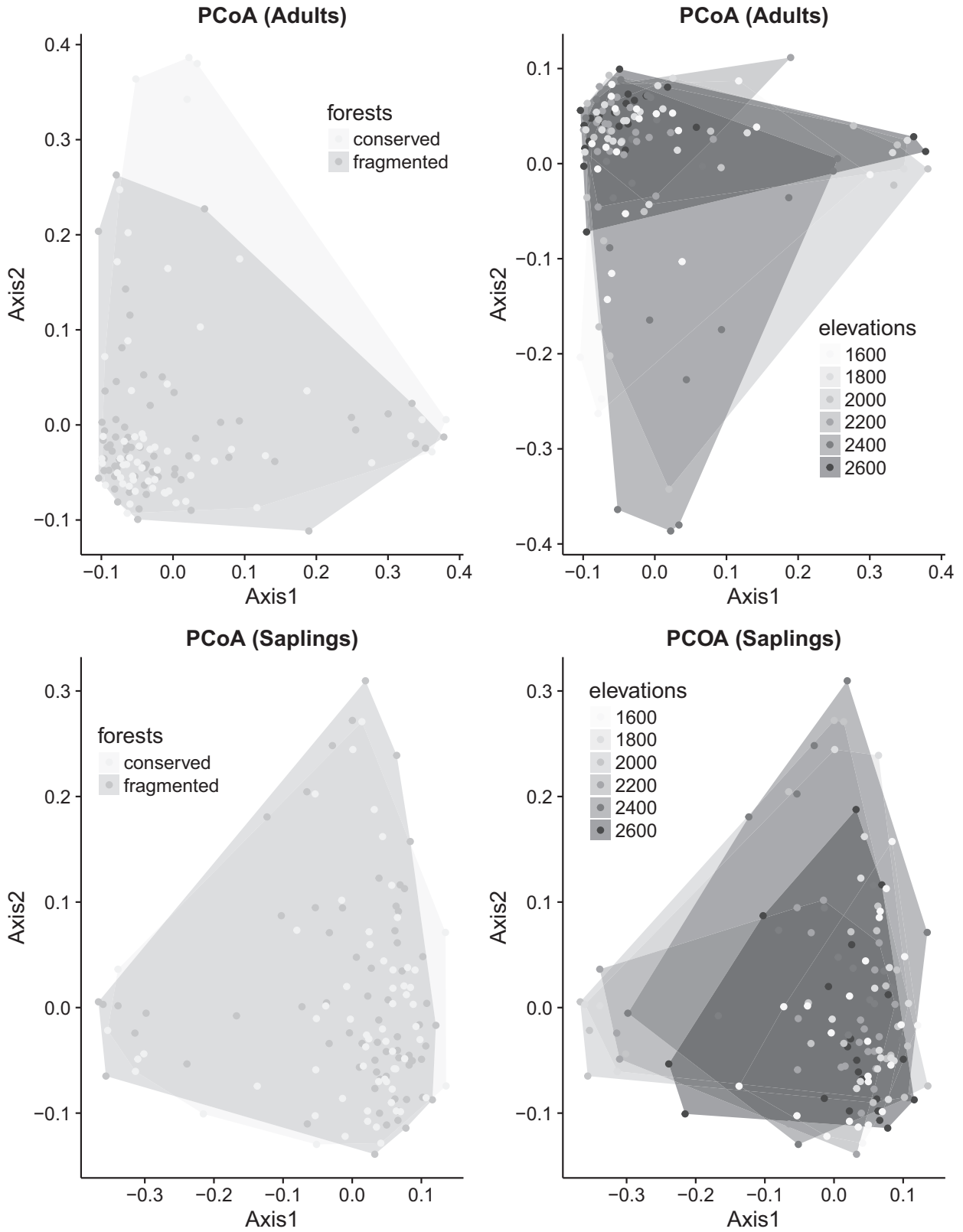


Fig. 2. PCoA from adults and saplings, for each forest transect (upper left and right, each shade of grey corresponds to a transect) and for each elevation (lower left and right, each shade of grey corresponds to an elevational band).

Table 3. Summary of Mantel and partial Mantel tests comparing geographic, elevational and genetic distance (F_{ST}) considering Pearson assumptions for adults and saplings of *Polylepis australis*

Matrix 1	Matrix 2	Control Matrix	r_M (A)	P (A)	r_M (S)	P (S)
Geographic	F_{ST}		-0.086	0.73	0.191	0.08
Elevation	F_{ST}		0.016	0.43	0.272	0.04
Geographic	F_{ST}	Elevation	-0.132	0.86	0.008	0.47
Elevation	F_{ST}	Geographic	0.103	0.22	0.197	0.10

A, Adults; S, Saplings. Significant value are in bold.

populations along elevational gradients (Table 2, Fig. 2). One possible reason is that *P. australis* genetic variation is more prevalent within populations (at least 98% for both forests) than among them. This pattern is consistent with previous studies for *P. australis* (Hensen *et al.* 2011; Julio *et al.* 2011), and it is in accordance with several reviews which showed that wind-pollinated tree species have a high proportion of genetic variability within populations and clearly lower diversity among populations (Hamrick 2004; Nybom 2004). The results confirm that extensive gene flow connects populations along the elevational gradient. In addition, as no elevational genetic structuring has been detected in *P. australis*, the study did not support the idea that local adaptation occurred along elevational gradients for *P. australis* populations characterized by phenological differences in flowering. It is more likely that phenotypic plasticity has resulted in phenological differences in flowering for this species. We propose that the studied *Polylepis* populations would be genetically connected throughout the elevational gradient due to their amplitude in flowering phenology which may be observed even within a single individual, which would compensate for average phenological differences in timing of flowering along the gradient.

Fragmentation

The levels of genetic differentiation revealed in our study were very similar between saplings and adults, indicating that fragmentation has not affected genetic diversity and differentiation in the studied species. This result is not in accordance with previous studies on populations of *P. incana* (Hensen *et al.* 2012) and the only other small population of *P. australis* studied (Julio *et al.* 2008), both of which showed significant differences in genetic differentiation between juvenile and adult trees. Our results are however consistent with other empirical studies on pollen flow in tree species in fragmented landscapes, all of which demonstrated robust pollen dispersal (White *et al.* 2002; Jha & Dick 2010). The results confirm

that tree populations may be resilient to the effects of habitat fragmentation because of the long generation time and extensive pollen dispersal of wind-pollinated trees even in fragmented habitats (Hamrick 2004; Kramer *et al.* 2008). However, negative effects on seed quality resulting from such fragmentation were found in *Polylepis* species (e.g. Seltmann *et al.* 2009). Previous studies of *P. besseri*, *P. incana*, *P. multijuga* and some populations of *P. australis* indicated that fragmentation resulted in low genetic diversity and strong genetic structure in these species (Table 3). The reasons for the maintenance of genetic connectivity could be due to extensive gene flow, species longevity and/or tetraploidy.

Possible conservation and restoration strategies

Based on the genetic evidence of our study on the joint effects of elevation and forest fragmentation, the two elevational forest transects in this study are not genetically structured along elevational gradients and they are still genetically unaffected by forest fragmentation, indicating that seeds for reforestation purposes could be collected everywhere in these two transects. This scenario probably holds true for other forests in the region, as the study transects represent the largest existing differences in altitudes for the species (Marcora *et al.* 2008). In addition, across the scale of our study, no spatial genetic structure was evident from isolation-by-distance patterns, and all populations were clustered together, indicating that the continuous and fragmented forests are probably relicts of an ancestral panmictic forest. This therefore suggests that, in terms of conservation, the studied forests may represent one genetic unit. In addition, there is no elevational genetic structuring, implying that seeds for forest restoration purposes or ex situ conservation could be collected from any elevational belt.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Quantitative characteristics of final peak selection from AFLP fingerprints in *Polylepsis australis* with four combinations.

Appendix S2 Pairwise F_{ST} between 12 populations.