Genetic and epigenetic diversity and structure of *Phragmites australis* from local habitats of the Songnen Prairie using amplified fragment length polymorphism markers

T. Qiu[1,2,3], L.L. Jiang[3] and Y.F. Yang[1]

[1]Key Laboratory of Vegetation Ecology, Ministry of Education, Institute of Grassland Science, Northeast Normal University, Changchun, Jilin, China
[2]School of Life Sciences, Changchun Normal University, Changchun, Jilin, China
[3]Key Laboratory of Molecular Epigenetics, Ministry of Education, Northeast Normal University, Changchun, Jilin, China

Corresponding author: Y.F. Yang
E-mail: yangyf601@yahoo.com

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ABSTRACT. The genetic and epigenetic diversity and structure of naturally occurring *Phragmites australis* populations occupying two different habitats on a small spatial scale in the Songnen Prairie in northeastern China were investigated by assessing amplified fragment length polymorphisms (AFLPs) and methylation-sensitive amplified polymorphisms (MSAPs) through fluorescent capillary detection. The two groups of *P. australis* were located in a seasonal waterlogged low-lying and alkalized meadow with a pH of 8-8.5 and in an alkaline patch

without accumulated rainwater and with a pH greater than 10. These groups showed high levels of genetic diversity at the habitat level based on the percentage of polymorphic bands (90.32, 82.56%), Nei's gene diversity index (0.262, 0.248), and the Shannon diversity index (0.407, 0.383). Although little is known about the between-habitat genetic differentiation of *P. australis* on a small spatial scale, our results implied significant genetic differentiation between habitats. Extensive epigenetic diversity within habitats, along with clear differentiation, was found. Specifically, the former habitat (Habitat 1, designated H1) harbored higher levels of genetic and epigenetic diversity than the latter (Habitat 2, designated H2), and population-level diversity was also high. This study represents one of few attempts to predict habitat-based genetic differentiation of reeds on a small scale. These assessments of genetic and epigenetic variation are integral aspects of molecular ecological studies on *P. australis*. Possible causes for within- and between-habitat genetic and epigenetic variations are discussed.

Key words: AFLP; MSAP; *Phragmites australis*; Genetic diversity; Genetic differentiation; Habitat

INTRODUCTION

Phragmites australis (Cav.) Trin. ex Steud. is a widespread wetland grass and clonal species typically occurring in marshes, which are the littoral zones of lakes and rivers. This species is also distributed in drylands and other areas subject to natural abiotic stresses (Lambertini et al., 2008; Kirk et al., 2011; Nada et al., 2015). The high degree of variability and genetic and life history traits of this species may contribute to its cosmopolitan distribution. As such, this species is distributed widely across the Songnen Prairie in northeastern China. *P. australis* often grows as a companion species in alkalized meadows but forms monodominant communities in local low-lying areas or saline-rich/alkaline patches (Li et al., 2009). This species is used as a major forage grass in the Songnen Prairie due to its high level of productivity and high protein content. In addition, it is an ecologically important habitat for ecological renovation and nutrient retention due to its perennial rhizome system and particular adaptation to stress conditions (Yang and Lang, 1998; Koppitz, 1999; Wu et al., 2013).

Among several molecular tools developed to elucidate the molecular ecology of perennial species, amplified fragment length polymorphism (AFLP) analysis has proven to be one of the most suitable and efficient genome-scanning approaches for revealing within- and between-population genetic diversity. This has been determined in various species, including *Leymus chinensis* (Gong et al., 2007), *Hordeum brevisubulatum* (Li et al., 2008), *Alternanthera philoxeroides* (Gao et al., 2010), *Fallopia japonica* (Richards et al., 2012), *Incarvillea younghusbandii* (Zhu et al., 2009), and *Viola cazorlensis* (Herrera and Bazaga, 2008). AFLP, methylation-sensitive amplified polymorphism (MSAP), and specific-sequence amplified polymorphism markers have also been used to analyze genetic/epigenetic diversity in wild barley (*H. brevisubulatum*) (Shan et al., 2012). In addition, fluorescent AFLP marker-based genotyping performed through capillary gel electrophoresis has improved upon the original AFLP technology and is considered to be a powerful and reliable technique due to its

high-resolving power, high sensitivity, and the requirement for small sample (Hasbún et al., 2012).

P. australis found in the Songnen Prairie of northeastern China are interesting because they have evolved easily discernable phenotypes in terms of their leaf, rhizome, and shoot morphology and biomass, which have adapted to various ecological habitats (Yang and Lang, 1998; Yang and Li, 2003). Among these, we focused on two groups of *P. australis* in two corresponding habitats, i.e., a seasonal waterlogged low-lying and alkalized meadow with a pH of 8-8.5 (Habitat 1, designated H1) and an alkaline patch without accumulated rainwater with a pH greater than 10 (Habitat 2, designated H2). These two habitats lie within a mosaic of contiguous diverse habitats in the grassland of the Songnen Prairie, which have arisen from severe soil salt-alkalization and vegetative deterioration, particularly during the last 50 years.

Processes resulting in the genetic structuring of plant populations generally include isolation-by-distance and habitat-based genetic differentiation (Mooney et al., 2010). However, little is known about the between-habitat genetic differentiation of *P. australis* on a small spatial scale (Pellegrin and Hauber, 1999; Guo et al., 2003; Gao et al., 2012). A preliminary investigation of the genetic diversity of this reed in the Songnen Prairie showed extensive genetic variability across a certain geographic range (Li et al., 2009). However, the within- and between-habitat genetic diversity and differentiation on a small spatial scale in the Songnen Prairie have not yet been studied. In addition to DNA sequence variation, epigenetic mechanisms can cause variation in gene expression and ecologically relevant morphological characteristics and, hence, are recognized as potentially important components of an individual's response to the environment. Variations in DNA methylation, which is the best described epigenetic mechanism and is often heritable in plants, are intriguing; however, only a few studies have used MSAPs, i.e., methylation-sensitive AFLPs to address research questions in wild plant populations (Schrey et al., 2013; Jiao et al., 2015).

In this study, the following issues were addressed to answer fundamental questions at the DNA level for *P. australis* plants growing in different habitats on a scale of approximately 10 x 5 km (Blondel et al., 1999). First, we assessed the levels of genetic diversity within habitats. Second, the degree of genetic differentiation in *P. australis* between habitats was evaluated. Third, because epigenetic states can be directly disrupted by the environment and can be inherited, epigenetic diversity and structure of populations were determined.

MATERIAL AND METHODS

Plant materials

Samples were collected from the Pasture Ecology Research Station of Northeast Normal University, Changling, Jilin Province, in the Songnen Prairie of China (123°45'E, 44°45'N). This region exhibits a temperate, semi-arid, and semi-wet monsoon climate. The two groups of *P. australis* are distributed in different habitats with distinct soil-water levels and pH values. In H1, the soil is alkalized meadow soil with a pH of 8-8.5, and water accumulates seasonally during the rainy season. The monodominant reed community grows well and plants are tall because of a plentiful water supply, providing coverage greater than 85%. In H2, the soil of alkaline patches is hardened with the top soil lost completely and exhibits a pH greater than 10. The reeds grow sparsely and in tufts with short stems as a monodominant species, providing community coverage of less than 20%. Each sampled group

occurs widely because these habitats lie within a mosaic of various habitats. Fully expanded fresh leaves were collected from 20 randomly selected individual plants of each group, which were distributed in the western, middle, and eastern areas of the research station, within a study area of approximately 10 x 5 km; thus, three populations were sampled within each habitat. All of the individuals were separated by at least 30 m to avoid sampling the same gene from several individuals. The numbers of individuals at each site were 6, 7, 7, and 7, 6, 7 in H1 and H2, respectively. Minimizing the impact of reed collection on local soil erosion was taken into consideration. The leaves were dried using abundant silica gel and were stored at -20°C prior to extraction.

AFLP genotyping and MSAP epi-genotyping

Genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) method. A standard AFLP analysis (Vos et al., 1995) was performed, with minor modifications (Wang et al., 2005) and technical improvements, which including the use of fluorescently labeled selective amplification primers (Applied Biosystems Inc., Foster City, CA, USA) and an ABI-automated 3730XL DNA capillary sequencer. The primers that provided the most reliable bands were selected based on the screening results (**Table S1**). MSAP was performed by following the AFLP protocol, except that the *Mse*I enzyme was replaced with either *Msp*I or *Hpa*II, which identify the same 5'-CCGG sequence but show differential levels of sensitivity to methylation. A ROX-500-labeled internal size standard (Applied Biosystems) was used for each sample. We utilized the GeneMapper v.4.1 software (Applied Biosystems) to collect and score the raw fluorescence data. Noisy sequencer electropherograms were obtained from one plant in several analyses, and this plant was subsequently excluded. We assessed the repeatability of banding patterns by performing two sets of independent AFLP analyses and including only the consistent bands.

All of the reliable bands were scored using the following binary code: 0 for an absent band and 1 for a present band. The markers showing only one non-consensus band were excluded from the data set. Only fragments ≥100 bp in size were scored to decrease the effect of size homoplasy. Three different types of MSAP banding patterns were scored, in which the fragments were 1) present in both the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I products, 2) absent in both the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I products, or 3) present only in the *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I product. The type-(1) pattern corresponds to a non-methylated state, whereas the type-(3) pattern indicates a methylated state, and the type-(2) pattern was treated as a missing score, possibly due to sequence mutation or hypermethylation. We classified each locus as either 'methylation-susceptible' or 'non-methylated' depending on whether the observed proportion of discordant *Msp*I/*Hpa*II scores exceeded a threshold that was set as the probability of a mismatch between the *Msp*I and *Hpa*II scores resulting from errors (Herrera and Bazaga, 2010). All of the scoring was conducted 'blindly', without any details on the samples being available.

Data analyses

The levels of genetic diversity within each habitat and population were calculated using the Popgene 1.32 programs (Yeh et al., 2000). The genetic diversity parameters determined included the number of alleles per locus (N_A), the percentage of polymorphic loci (*P*), Nei's

gene diversity index (H_E), and Shannon information index (I).

Genetic and epigenetic structure was evaluated through analysis of molecular variance (AMOVA) in which the allocation of variance between habitats, among sites, i.e., populations, and within populations was estimated with GENALEX 6.5, using 999 random permutations (Peakall and Smouse, 2006). We employed the msap package in R to analyze the MSAP results (Pérez-Figueroa, 2013). We also conducted a locus-by-locus AMOVA to characterize the epigenetic differentiation at each msap locus using GENALEX.

Genetic relationships between habitats were determined from the values for Nei's genetic distance and the estimate of gene flow (N_m) obtained using Popgene, and by utilizing the analytical methods mentioned above. The relationships among different individuals were investigated using the principal coordinate analysis (PCoA) program in the msap package to analyze the AFLP data (Pérez-Figueroa, 2013). The Student t-test was used to test for differences in N_A, I and H_E between the habitats at $\alpha = 0.05$.

RESULTS

Genetic diversity

Four AFLP primer pairs produced 992 reliable fragments, with a relatively low scoring error rate. The number of bands per primer pair ranged from 161 to 331 according to the efficiency of each primer, and the levels of polymorphism were high, ranging from 72.63 to 87.01% (Table 1). At the intra-habitat level, the two groups of *P. australis* showed high levels of genetic diversity. The *P* bands in H1 and H2 were 90.32 and 82.56%, respectively. Likewise, the mean N_A were 1.903 and 1.826; the H_E indices were 0.262 and 0.248; and the I indices were 0.407 and 0.383, respectively, for the reeds in H1 and H2 (Table 2). At the population level, the data also showed high levels of genetic diversity according to the average N_A (1.667, 1.574), H_E (0.2332, 0.2087), I (0.3516, 0.3128), and P (66.73, 57.36%) in H1 and H2, respectively.

Table 1. Primer combinations, number of markers, observed levels of polymorphism, and estimates of scoring error rates in the amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) analyses.

Primer combination	Total bands	Polymorphic bands[1]		Polymorphic rate (%)		Scoring error rate (%)[2]		
A. AFLP analysis								
1. 4b	190	138		72.63		0.936		
2. 4i	331	288		87.01		1.11		
3. 7a	161	131		81.37		0.676		
4. 3c	310	249		80.32		1.15		
		Non-methylated markers		Methylation-susceptible markers				Estimated probability of errors
		N	Polymorphic rate (%)[3]	N	Polymorphic rate (%)[4]	*Hpa*II	*MspI*	*Hpa*II-*Msp*I mismatch[5]
B. MSAP analysis								
1. 5h	318	94	85.11	224	80.80	1.40	1.29	0.0265
2. 3g	299	101	88.12	198	88.38	1.26	1.17	0.0240

[1]Number of polymorphic bands at the 5% level. [2]Calculated from six individual plants that were re-assayed as 100X (number of discordant scores in two independent analyses) / (number of scored markers x number of individuals). [3]A non-methylated marker was considered polymorphic if at least two individuals in the sample displayed a variant score. [4]A methylation-susceptible marker was considered polymorphic when both the methylated and non-methylated states occurred in the studied individuals. [5]Estimated average probability of obtaining discordant *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I scores estimated from the scoring error rates for *Eco*RI-*Hpa*II ($=e_{Hpa}$) and *Eco*RI-*Msp*I ($=e_{Msp}$) as $e_{Hpa}+e_{Msp}-2e_{Hpa}e_{Msp}$.

Table 2. Genetic diversity in *Phragmites australis* from two habitats (H1; H2) using AFLP analysis.

	N_A	H_E	*I*-AFLP	*P*-AFLP	N_m	Genetic distance
H1	1.903	0.2621	0.4074	90.32%		
H2	1.826	0.2480	0.3830	82.56%		
P value	<0.001*	0.007*	0.001*			
					8.177	0.04270
H1 population mean	1.667	0.2332	0.3516	66.73%		
H2 population mean	1.574	0.2087	0.3128	57.36%		

Gene flow (N_m), genetic distance, and *t*-tests between habitats are shown. N_A, mean number of alleles per locus. H_E, Nei's gene diversity. *I*, Shannon information index. *P*, percentage of all loci that are polymorphic regardless of allele frequencies. N_m = estimate of gene flow from G_{ST}, e.g., $N_m = 0.5\ (1\text{-}G_{ST}) / G_{ST}$. P values for *t*-tests of N_A, H_E and *I* at $\alpha = 0.05$.

Epigenetic diversity

Among the 617 MSAP loci observed, 422 were methylation-susceptible, 80.80-88.38% of which were polymorphic, and 195 loci were non-methylated loci, 85.11-88.12% of which were polymorphic (Table 1). At the intra-habitat level, 13.85 and 12.71% of the methylation-susceptible were externally cytosine hemimethylated, and 14.91 and 15.02% were internally cytosine methylated; 21.19 and 22.29% were unmethylated while the remaining 50.05 and 49.99% were fully methylated or lost the restriction site resulting from sequence mutation, in H1 and H2, respectively. These results suggested that the levels of internal cytosine methylation were higher than the levels of external cytosine hemimethylation, and the total methylation level in *P. australis* was approximately 28% in both habitats. Approximately 91.41 and 84.93% of the bands observed in samples from H1 and H2 were polymorphic, with *I* indices of 0.4245 and 0.3984, respectively (Table 3). At the population level, the average values for *P* were 70.66 and 61.59%, and for *I* were 0.3712 and 0.3381 in samples from H1 and H2, respectively.

Genetic and epigenetic structure

Hierarchical AMOVA of AFLP loci revealed that 5% of the total molecular variance was attributable to significant differentiation between habitats ($P < 0.001$), 6% was attributable to significant differentiation among populations ($P < 0.001$), and 89% was attributable to that within populations ($P < 0.001$). The value of Φ_{PT}, which is a measure of population genetic differentiation for binary data and is analogous to F_{ST}, was 0.105. The genetic distance and the value for gene-flow (N_m) between the habitats were 0.04270 and 8.177, respectively. Genetic relationships among individuals and between habitats could be summarized by PCoA, which showed a certain degree of genetic subdivision between the habitats (Figure 1). The *t*-tests, performed to compare genetic diversity, revealed that N_A ($P < 0.001$), H_E ($P = 0.007$), and *I* ($P = 0.001$) were significantly different between the habitats (Table 2). The AMOVA-based MSAP estimate showed that significant differentiation was present between habitats ($P < 0.001$), among populations ($P < 0.001$), and within populations ($P < 0.001$), accounting for 2, 4, and 94% of the total molecular variance, respectively (Table 4). The value for Φ_{PT} was 0.06. The *t*-tests performed to compare epigenetic diversity between the habitats showed that values for *I* ($P = 0.001$) were significantly different (Table 3). A locus-by-locus AMOVA showed that 26 MSAP loci were significantly differentiated (**Table S2**). The level of external cytosine hemimethylation for *P. australis* in H1 was 1% more than that for plants from H2

whereas the level of the unmethylated in H1 was 1% less than that in H2. Interestingly, all of the parameters related to the genetic and epigenetic variability of the habitats showed higher values in H1 than in H2.

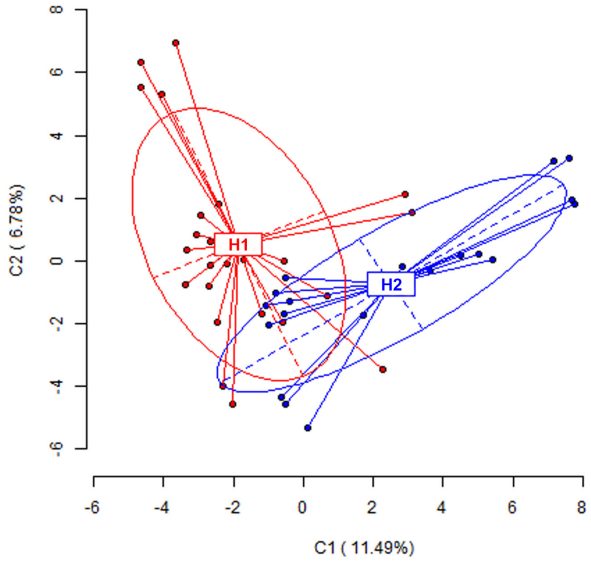

Figure 1. Principal coordinate analysis (PCoA) based on AFLP banding patterns showing the relationships among individuals from two habitats. H1 denotes habitat 1; H2 denotes habitat 2.

Table 3. Epigenetic diversity and methylation levels in *Phragmites australis* from two habitats (H1; H2) using MSAP analysis.

	P-MSAP	*I*-MSAP	Methylation levels			
			Hemimethylated	Internal cytosine methylation	Non-methylated	Full methylation or absence of target
H1	91.41%	0.4245	13.85%	14.91%	21.19%	50.05%
H2	84.93%	0.3984	12.71%	15.02%	22.29%	49.99%
P value		0.001*				
H1 population mean	70.66%	0.3712				
H2 population mean	61.59%	0.3381				

P, percentage of all loci that are polymorphic regardless of allele frequencies. *I*, Shannon information index.

Table 4. Results of AMOVA within and between *Phragmites australis* populations from two habitats, i.e., the seasonal waterlogged low-lying meadow (H1) and alkaline patch without seasonally accumulated rainwater (H2) for (A) AFLP and (B) MSAP.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	P value
A. AFLP					
Among habitats	1	339.690	6.908	5	<0.001
Among populations	4	749.763	8.108	6	<0.001
Within populations	39	4967.836	127.380	89	<0.001
Total	44	6057.289	142.396		
B. MSAP					
Among habitats	1	205.536	2.608	2	<0.001
Among populations	4	591.787	4.608	4	<0.001
Within populations	39	4438.676	113.812	94	<0.001
Total	44	5236.000	121.028		

DISCUSSION

We found that levels of genetic polymorphism within habitats and populations were generally high. The mean diversity indices for population-level variation (P, H_E; Table 2) of *P. australis* were higher than those reported for long-lived perennial herbaceous plants (P = 39.3%, H_E = 0.084) by Hamrick and Godt (1990). A comparison of our results (N_A, P, H_E; Table 2) with a summary of 56 mostly terrestrial sexual and asexual species showed that the values for *P. australis* in the present study were higher than those for the 56 species in the previous study (N_A = 1.47, P = 29.4%, H_E = 0.103) (Hamrick and Godt, 1990; Pellegrin and Hauber, 1999). Moreover, genetic variation within populations explained more of the total variation than that between the populations (Table 4). Levels of genetic diversity and its partitioning are correlated with the activity of the reproductive system. Although the reproductive biology of *P. australis* has not been fully resolved, it is clear that *Phragmites* reproduce both clonally and sexually and are self-compatible. Generally, cross-pollinating plant species show high levels of genetic variation within populations but low levels of divergence between populations, whereas self-pollinating or asexually reproducing species show the opposite pattern (Mateu-Andrés and de Paco, 2006; Kirk et al., 2011). Therefore, our results indicated that sexual reproduction occurs more frequently than previously thought in *P. australis*. In light of the clear genetic differentiation between populations, inbreeding or clonal reproduction may also have occurred. It has been proposed that *P. australis* can disperse over short and long distances via rhizome fragments or seeds. In addition to dispersal via air or water, seeds may also be ingested and dispersed by herbivores or they may stick to the feathers of migratory birds. Pollen can be carried over long distances by air, particularly during the autumn monsoon in the Songnen Prairie. Because *P. australis* typically displays clonal growth, somatic mutations, which are maintained within populations, may bea plausible explanation for the high observed genetic diversity (Guo et al., 2003). Population history is another possible factor leading to high levels of genetic polymorphism. *P. australis* is thought to have been native to the Songnen Prairie as long ago as the early Pleistocene. Historical climatic fluctuation and neotectonic activity could have driven frequent extinctions and recolonizations of local populations, which may have led to multiple founding genotypes or to sexual reproduction between new colonizers and other resident *Phragmites* genotypes (Zhang and Wang, 2001; Kirk et al., 2011).

We found that the level of genetic differentiation between the habitats is still high. The results of a previous study showed that the average genetic distances between clones from the Po Plain in Italy and geographically distant clones located 1500-2000 or 2000-2530 km away in several Eurasian countries were 0.042 and 0.044, respectively (Lambertini et al., 2008). The genetic distance observed in our study was 0.0427, which is similar to the values found in that study. Thus, this suggested that the level of genetic differentiation is generally high, given that our study was conducted within an area of 10 x 5 km. There are several possible explanations for the pattern of genetic structure observed in the present study, including genetic drift, habitat induction, and mutation. However, it is highly unlikely that genetic drift resulted in this pattern because estimation of the number of migrants (N_m) gave a relatively high value, and there was substantial between-habitat variance, as demonstrated using the Student *t*-test and AMOVA. A possible explanation for the minimal genetic differences observed in the present study could be the presence of somatic mutations, which are known to occur in several clonal species, including reeds (Connor et al., 1998). It is most likely that habitat selection pressure (i.e., differential alkaline/saline conditions) may have played a major role in the observed genetic

diversity. As Kawecki and Ebert (2004) noted, local adaptation is a common result of natural selection on a small spatial scale, even under conditions of high gene flow (Kawecki and Ebert, 2004). In spite of the homogenizing effect of gene-flow between populations over small scales, there are some examples that indicate habitat-based genetic differentiation, supporting the so-called 'divergence-with-gene-flow' model (Shapiro et al., 2006; Mooney et al., 2010).

Assessments of the spectra and structures of epigenetic variation are integral aspects of molecular ecological studies, although few such assessments have been performed on wild plant populations. Because variations in DNA methylation are often heritable and could affect phenotypic outcomes or even evolution (Diez et al., 2014), we examined the epigenetic diversity and structure of *P. australis* in two habitats. Apparently, there is extensive within-population variance due to individual epigenotypic variation, a critical prerequisite for showing microevolutionary potential. Significant epigenetic differentiation presumably underlies these two distinct phenotypes occupying different habitats.

All of the genetic and epigenetic diversity indices assessed in the present study showed the same result, indicating that *P. australis* in H1 harbor a higher level of genetic and epigenetic diversity than those in H2, which is congruent with the niche-width variation hypothesis (Valen, 1965). Populations growing in a broader niche (e.g., a more diversified microgeographic habitat, such as H1) are hypothesized to exhibit more variability than populations inhabiting a narrower niche (such as H2).

Taken together, the results of the present study have greatly enhanced our understanding of the genetic and epigenetic basis of *P. australis* from local habitats on a small spatial scale. Accordingly, knowledge gained from genome scans of *P. australis* may provide insight or guidelines for studies of ecological adaptation and for conservation and utilization practices, including ecological revegetation and genetic improvement.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Adaptors, pre-selective primers, and primer pairs used for selective amplification in the amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) analyses.

Table S2. Summary of significant locus-by-locus tests of differentiation between the habitats for MSAP. An asterisk denotes significant locus-by-locus tests after sequential Bonferroni correction.