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Genetic Variation in the Qinghai-Tibetan Plateau Endemic and Endangered Conifer *Cupressus gigantea*, Detected Using RAPD and ISSR Markers

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Abstract

Assessing the level and distribution of genetic diversity of rare tree species is essential for their management and the development of effective conservation strategies. *Cupressus gigantea* is a long-lived endemic cypress of the west Qinghai-Tibetan Plateau and the tallest tree in its genus. The current populations of this species are fragmented and highly disturbed. We used RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat amplification) markers to assess the genetic variation and population structure of this

endangered cypress. The 15 RAPD primers used in this study amplified 108 reproducible bands, 49 (45.4%) of which were polymorphic, while the 12 ISSR primers amplified 94 bands, 65 (69.2%) of which were polymorphic. Analysis of Molecular Variance (AMOVA) indicated that 49.7% and 38.3% of the variation was attributable to differences between populations for the RAPD and ISSR markers, respectively; relatively high compared to values reported for other conifer species. These estimates were also similar to *Gst* values obtained from Nei's gene diversity analyses (RAPD = 0.41 and ISSR = 0.36), and suggest that there is a high degree of population differentiation in this narrowly-distributed conifer. The genetic structure of this species has probably been shaped by its long life cycle and climatic changes during the Quaternary. The high degree of population differentiation in this species highlights the need for additional conservation in this species, including measures to protect of all of the remaining populations. The substantial similarities between the results of the RAPD and ISSR analyses of samples from the same individuals indicate that they can be interpreted with high levels of confidence.

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Key words: *Cupressus gigantea*, genetic diversity, ISSR, RAPD, Qinghai-Tibetan Plateau.

Introduction

Genetic diversity provides the potential for tree species to adapt to long-term environmental change (HEDRICK, 2004). An overall loss of genetic variability usually has deleterious effects on species fitness and may threaten the ability of populations to survive and persist via natural regeneration (REED, 2003; KREMER and REVIRON, 2004). Assessing the level and distribution of genetic diversity within tree species is crucial for their management and the development of effective conservation strategies, especially for rare species (HEDRICK, 2004). A large number of studies focusing on the pattern and distribution of genetic variation within coniferous tree species have suggested that they have relatively high levels of genetic variation, with little differentiation among populations, compared with other plant taxa (HAMRICK et al., 1992; PETIT et al., 2004, 2005). This is mainly because most conifers have widespread distributions, outcrossing breeding systems, and relatively high potential for long-distance gene flow. However, exceptions have been recorded for a limited number of endangered coniferous species. For instance, low genetic diversity has been detected in *Abies bracteata* (D. Don) D. Don ex Poit (LEDIG, 1987) and *Calocedrus macrolepis* Kurz (WANG et al., 2004), while low intraspecific variation and high population differentiation has been found in *Juniperus thurifera* Linn. (JIMÉNEZ et al., 2003). These exceptional patterns are thought to be consequences of genetic bottlenecks when populations were

reduced in size during the Quaternary glaciations and founder effects during postglacial re-colonizations (e.g. HAMRICK et al., 1992; JIMÉNEZ et al., 2003; WANG et al., 2004; ALLNUTT et al., 2003; COTTRELL et al., 2005; PETIT et al., 2005; ZHANG et al., 2005).

Cupressus gigantea CHENG and FU occurs sparsely along 200 kilometres of the Yarlung (Zangbo) river at altitudes ranging between 3000 m and 3400 m, on the Qinghai-Tibetan Plateau (QTP), southeast Tibet (FU et al., 1999). This species is listed as threatened, with several relict populations forming pure stands (FU, 1995). *C. gigantea* individuals can reach diameters up to 6 m, heights exceeding 30 m and can live for more than 2000 years in the Bajie population near Linzhi county (FU et al., 1999). These are the tallest known members of the Cupressaceae in the world. According to the IUCN categories of threat, this species is classified as range-restricted and vulnerable (FU et al., 1999). However, only one population in Linzhi has been protected by the designation of a local "natural protection" region, and most materials used in artificial regeneration programs conducted by the local governments have originated from this population (NAEP and IBCAS, 1987; FU et al., 1999). Furthermore, despite the threatened status of this species, little is known about the genetic variation within and between its populations.

In the study presented here we used Randomly Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers to estimate the amount and partitioning of genetic diversity between populations in this species. RAPDs are being increasingly used to assess genetic variation in tree species (ALL-

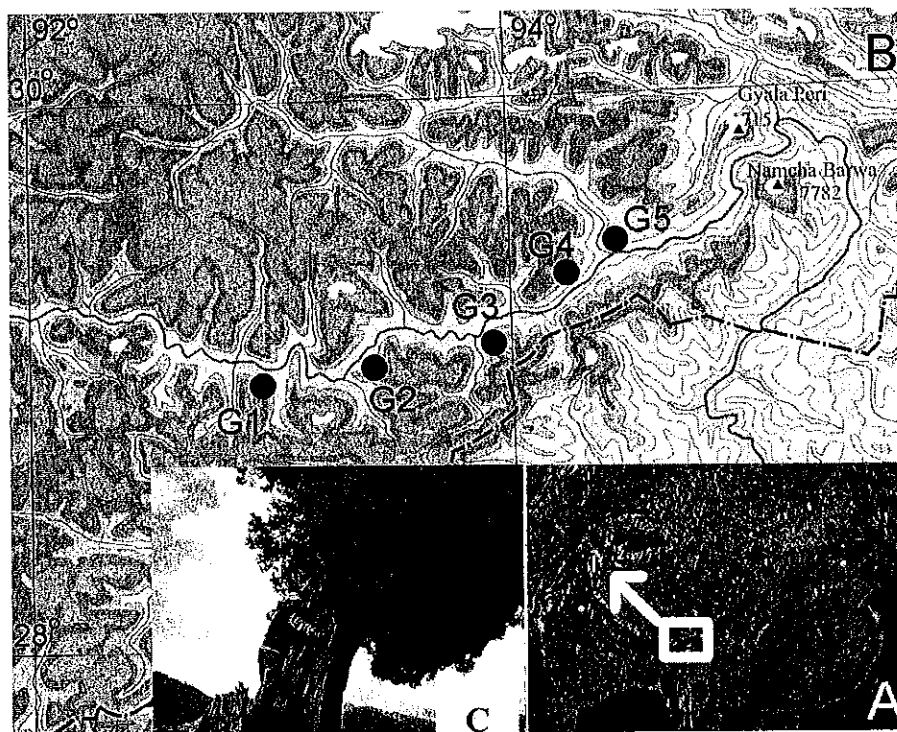


Figure 1. – Maps showing sampling locations (A) and (B), and photograph showing gross morphology of *Cupressus gigantea* (C).

NUTT et al., 1999, 2003; BEKESSY et al., 2002; GILLIES et al., 1997, 1999) because DNA sequence information is not required prior to investigating a previously unstudied species using them, which facilitates their application to a wide range of taxa (NEWTON et al., 1999; WANG and SZMIDT, 2001). However, RAPD markers have several limitations including dominance, uncertain locus homology, and their sensitivity to PCR reaction conditions. As dominant markers, ISSRs target simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome, evolve rapidly, and thus provide a much larger number of polymorphic fragments per primer than RAPDs. Since no prior knowledge of the DNA sequence is required to design primers (WOLFE et al., 1998), ISSR markers have recently been used to assess the levels of genetic diversity of some coniferous trees (e.g. WANG et al., 2004). Similar levels of genetic diversity and its partitioning among populations of a herb species have been found using both RAPD and ISSR markers (QIAN et al., 2001; NYBOM, 2004). However, results obtained by RAPD and ISSR analyses of populations of single coniferous tree species have not been directly compared in any published studies to date. The main objectives of this study were to address the following questions. How is the genetic diversity partitioned within and among populations of *C. gigantea*? Do the results obtained by analyses of the genetic structure of a species using RAPD and ISSR markers significantly differ, and if so, which marker is the more informative?

Methods

Sample collection

The distribution range of *C. gigantea* was investigated in 2001 and 2002. Three large populations containing more than 1000 individuals were found and at least 20 individuals from each of these populations were sampled (Table 1). However, two populations from Lang County contained less than 100 individuals, so we collected only 11 and six trees respectively from these two populations in order to retain a minimum sampling distance of 100 m between the sampled trees. These populations covered the total distribution range of this species along the Yarlung (Zangbo) river (Figure 1). All of the voucher specimens were deposited in the herbarium of the Northwest Plateau Institute of Biology, the Chinese Academy of Sciences, Xining, Qinghai, China.

DNA extraction and PCR reactions

All DNA was extracted according to the protocol described by DOYLE and DOYLE (1987). Initially 60 random decamer primers (Sangon Technologies, Shanghai, China) and 100 ISSR primers (UBC, Vancouver, Canada) were screened, but only 15 RAPD and 12 ISSR primers (Table 2) were used in the final analyses. These primers were chosen because they generated clear, reproducible banding patterns. We used the same PCR reaction kits, PCR machine and electrophoresis equipment for all the amplifications to maximize the uniformity.

Table 1. – The locations of the sampled *Cupressus gigantea* populations and the number of plants sampled at each site (N).

Population	N	Region	Altitude (m)	Latitude (N)	Longitude (E)
G1	23	Langri County	3200	29°02'29"	93°03'23"
G2	11	Lang County	3060	28°59'57"	93°14'06"
G3	6	Lang County	3050	29°01'19"	93°53'02"
G4	23	Milin County	2950	29°20'24"	94°22'38"
G5	23	Linzhi County	3040	29°40'	94°20'

Table 2. – Primers used for RAPD and ISSR marker amplification.

Primers	RAPD			ISSR ^a			
	Sequence (5'–3')	Primer	Sequence (5'–3')	Primers	Sequence (5'–3')	Primers	Sequence (5'–3')
AA-3	TCTCGCCTAC	AG-10	ACTGCCCGAC	UBC-807	(AG) ₈ T	UBC-824	(TC) ₈ G
AA-11	CAATCGGGTC	AG-14	CTCTCGGCGA	UBC-808	(AG) ₈ C	UBC-841	(GA) ₈ YC
AA-16	AACGGCCTAC	AG-20	TGCGCTCCTC	UBC-811	(GA) ₈ C	UBC-847	(CA) ₈ RC
AA-17	AGCGGAAGTG	G-01	CAACGGAGGA	UBC-812	(GA) ₈ A	UBC-848	(CA) ₈ RG
AG-4	GGAGCGTACT	G-07	GAACCTGCGG	UBC-815	(CT) ₈ G	UBC-857	(AC) ₈ YG
AG-7	CACAGACCTG	H-01	GGTCGGAGAA	UBC-818	(CA) ₈ G	UBC-858	(TG) ₈ RT
AG-8	AAGAGCCCTC	H-10	CCTACGTCAG				
AG-9	CCGAGGGGTT						

^a) Y: C/T; R: A/G

mity of the reaction conditions and minimize systematic errors.

All reactions were performed in a Tpersonal 20 thermocycler (Whatman Inc., Germany). The thermal program used for RAPD amplifications was 5 min at 94°C followed by 36 cycles of 50 sec at 94°C, 80 sec at 37°C, 120 sec at 72°C, and a final extension step of 7 min at 72°C. Reactions were performed in final volumes of 20 µl, containing 50 mM Tris-HCl (pH 8.3), 500 µg/ml BSA, 10% Ficoll, 1 mM tartrazine, 1.5 mM MgCl₂, 200 µM dNTPs, 1.0 µM of each primer, approx. 100 ng of DNA template, 1U *Taq* polymerase and 4% DMSO. The thermal program for ISSR amplifications was an initial 5 min at 94°C, followed by 38 cycles of 30 sec at 94°C, 45 sec at 50°C, 1 min 30 sec at 72°C, and a final extension step of 7 min at 72°C. Reactions were carried out in volumes of 20 µl, containing 50 mM Tris-HCl (pH 8.3), 500 µg ml⁻¹ BSA, 10% Ficoll, 1 mM tartrazine, 2 mM MgCl₂, 200 µM dNTP, 0.9 µM of each primer, 20–100 ng of DNA template, 1U *Taq* polymerase and 4% DMSO. All amplification products were separated by electrophoresis in a 2.0% agarose gel in 1 × TBE buffer, stained with ethidium bromide and viewed on a UV transilluminator (Bio-Rad). The molecular weights of the bands were estimated using a 100 bp DNA ladder (Casarray, Shanghai, China).

Data analysis

Only clear, polymorphic fragments that could be scored across all population samples were used in our analysis. These fragments were scored independently as present (1) or absent (0) in each population, and a binary data matrix was constructed. Both RAPD and ISSR fragments were analyzed as alleles, under the three following assumptions: RAPD products segregate as dominant alleles in a Mendelian fashion; the populations in this study were in Hardy-Weinberg equilibrium; the RAPD fragments represent the nuclear genome and fragments of the same apparent size in different trees are homologous. We used POPGENE version 1.31 (YEH et al., 1999) to calculate a set of intra- and inter-population genetic parameters, including genetic diversity within populations (H_s), genetic diversity between populations (D_{ST}) and the relative magnitude of genetic differentiation among populations ($G_{ST} = H_T - H_s/H_T$). Nei's gene diversity (H_e) was also calculated (NEI, 1973).

Shannon's indices (LEWONTIN, 1972) were also calculated and used to characterize the gene diversity and distribution of the variation, based on the formula $H_0 = -\sum p_i \log_2 p_i$, in which p_i is the frequency of a given RAPD fragment. H_0 was calculated at two levels: the average diversity within populations (H_{pop}) and the total diversity (H_{sp}). The proportion of diversity among populations was estimated as $(H_{sp}-H_{pop})/H_{sp}$.

Analysis of Molecular Variance (AMOVA, EXCOFFIER et al., 1992) was performed on a matrix of squared standard Euclidean distances. Input data files for the AMOVA version 1.55 program (EXCOFFIER et al., 1992) were generated using AMOVA-PREP (MILLER, 1998). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations. To examine the genetic relationship among populations, a dendrogram was also constructed using the unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) with the computer programme NTSYSpc version 2.02c (ROHLF, 1997).

Results

Genetic diversity

Of the 50 RAPD primers screened, 15 produced clear RAPD patterns. The screening of the entire sample set was repeated with two of the primers to assess the repeatability of the RAPD profiles, and we obtained identical RAPD patterns. One hundred and eight clear and repeatable RAPD patterns were scored. The average number of bands was 8.3 per primer. Of the 108 RAPD bands scored, 49 (45.4%) were polymorphic, based on the 95% criterion. The total gene diversity at the species level (H_t) and Nei's gene diversity (H_e) were estimated to be 0.124 and 0.121. The Shannon indices (H_{pop}) of the *C. gigantea* populations ranged from 0.08 to 0.13, with an average of 0.10 at the population level (H_{pop}) and 0.18 at the species level (H_{sp}).

Screening of the entire set of samples using the 12 selected ISSR primers generated a total of 94 bands ranging in length from 230 to 1500 bp, corresponding to an average of 10.6 bands per primer, of which 65 were polymorphic (69.2%). The total gene diversity at the species level (H_t) and Nei's gene diversity (H_e) were estimated to be 0.19 and 0.20. The Shannon indices (H_{pop}) for the populations ranged from 0.15 to 0.21, with an

Table 3. – Genetic variation in five populations of *Cupressus gigantea*.

Population	RAPD					ISSR				
	N _B	N _{PB}	PPB %	H _e	H _{pop}	N _B	N _{PB}	PPB %	H _e	H _{pop}
G1	100	33	33.00	0.0644	0.0989	82	40	48.78	0.1280	0.1882
G2	98	37	37.76	0.0845	0.1277	87	46	52.87	0.1335	0.1993
G3	91	27	29.67	0.0684	0.1002	83	44	53.01	0.1422	0.2068
G4	97	30	30.92	0.0544	0.0845	80	37	46.25	0.1019	0.1532
G5	96	28	29.17	0.0570	0.0869	88	48	54.55	0.1187	0.1833
Total	108	49	45.37	0.1160	0.1762	94	65	69.15	0.1892	0.2847

N_B = total number of bands; N_{PB} = number of polymorphic bands; P_{PB} = percentage of polymorphic bands; H_e = Nei's (1973) gene diversity or expected heterozygosity; H_{pop} = Shannon's information index (LEWONTIN, 1972) or observed heterozygosity.

Table 4. – Matrix based on Nei's (1973) pairwise genetic distances between *Cupressus gigantea* populations obtained using ISSR (above diagonal) and RAPD (below diagonal) marker data.

Population	G1	G2	G3	G4	G5
G1	***	0.3761	0.3853	0.4700	0.4276
G2	0.3135	***	0.2045	0.6480	0.5704
G3	0.3197	0.1850	***	0.5178	0.5038
G4	0.3750	0.4769	0.4042	***	0.6423
G5	0.3479	0.4347	0.3958	0.4739	***

Table 5. – Analysis of Molecular Variance (AMOVA) for *Cupressus gigantea* populations using RAPD and ISSR markers.

Source of variation	d.f	SSD	MSD	VC ^a	TV(%) ^b	P – value ^c
RAPD						
Among populations	4	346.5260	86.631	4.967	49.73	< 0.0010
Among individuals	81	406.6601	5.020	5.020	50.27	
ISSR						
Among populations	4	385.1335	96.283	5.337	38.34	< 0.0010
Among individuals	81	695.4130	8.585	8.585	61.66	

^{a)} Variance components; ^{b)} Percentage of total variance; ^{c)} P – value = probabilities calculated from 1000 random permutations of individuals across the populations.

average of 0.19 at the population level (H_{pop}) and 0.28 at the species level (H_{sp}). Genetic distances of paired populations calculated from ISSR and RAPD markers are shown in Table 4. All estimated genetic parameters were higher for ISSR markers than for RAPDs (Tables 2, 3 and 4).

Genetic partitioning among populations

Both RAPD and ISSR markers revealed similar, high levels of genetic differentiation among populations. The coefficients of overall genetic differentiation between populations (G_{st} ; estimated by the partitioning of the total gene diversity) were calculated as 40.2% and 36.3% for RAPD and ISSR markers, respectively. AMOVA analyses (Table 5) also suggested that there were significant ($P < 0.001$) genetic differences (Φ_{ST}) among the five populations; 49.7% (RAPDs) and 38.3% (ISSRs) of which was attributable to among-population variation and the rest to differences within populations (Table 5). The UPGMA dendrograms, based on the genetic distances between populations for ISSR and RAPD markers, are shown in Figure 2. For RAPDs,

there are two distinct groups, Populations G2 and G3; and Populations G1, G4 and G5. In contrast, there is less of distinct grouping with the ISSR data. However, other than the two distinct groupings, the results are similar since in the ISSR data, populations G5, G4, G1 seem to be distinctly from one another and G2 and G3 are closely related.

Discussion

Genetic partitioning within and among populations

The genetic variation of *C. gigantea* (45.4% and 69.2% of the total amplified bands were polymorphic for RAPD and ISSR markers, respectively) was similar to, or lower than, that reported for other conifers (JIMÉNEZ et al., 2003; WANG et al., 2004; ALLNUT et al., 2003; COTTRELL et al., 2005; PETIT et al., 2005; ZHANG et al., 2005). To our surprise, AMOVA indicated that 49.7% and 38.3% of the total variation recorded for RAPD and ISSR markers, respectively, was partitioned among populations and was consistent with Nei's genetic diversity analyses ($G_{ST} = 0.41$, RAPD and $G_{ST} = 0.36$, ISSR). These esti-

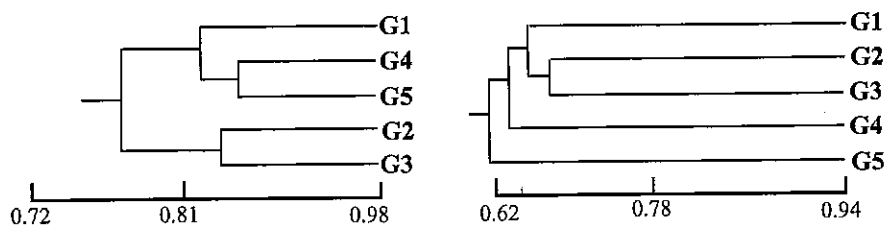


Figure 2. – UPGMA dendrogram (all individuals in one population form a single cluster) illustrating the genetic relationships between all populations of *Cupressus gigantea* calculated from the RAPD (left) and ISSR marker data (right).

mates of differentiation among populations are much higher than corresponding coefficients reported for other conifer species of the same family based on estimates using RAPD or ISSR markers, e.g. *Pilgerodendron uviferum* ($\Phi_{ST} = 0.186$, RAPD; ALLNUTT et al., 2003), *Araucaria araucana* (Molina) K. Koch. ($\Phi_{ST} = 0.128$, RAPD, BEKESSY et al., 2002), *Fitzroya cupressoides* (Molina) I.M. Johnston ($\Phi_{ST} = 0.144$, RAPD, ALLNUTT et al., 1999), *Podocarpus salignus* D. Don. ($\Phi_{ST} = 0.07$, RAPD, ALLNUTT et al., 1999), and *Calocedrus macrolepis* ($\Phi_{ST} = 0.04$, ISSR; WANG et al., 2004). Most gymnosperm species have low genetic differentiation among populations (similar to our results). HAMRICK and GODT (1989) reported a mean *Gst* value of 0.07 for 121 conifer species they examined, based on allozyme analyses. This tendency for gymnosperms to display low levels of population differentiation has been further confirmed by NYBOM and BARTISH (2000) based on a recent overview of RAPD analyses of plant species. Interspecific differences in the levels of among-population differentiation have been attributed to differences in breeding systems, life-history traits (NYBOM and BARTISH, 2000) and geographical distribution (LEE et al., 2002). However, compared with the above-listed conifer species from the same family, *C. gigantea* shows no distinct difference in its breeding system or life-history traits, except for a longer life cycle. In addition, all the investigated conifers mentioned above, including *C. gigantea*, are narrowly distributed with restricted ranges.

The climatic oscillations that occurred across the world during the Quaternary are considered to have strongly affected the distribution ranges of tree species (DUMOLIN-LAPEÁ GUE et al., 1997; NEWTON et al., 1999; PETTIT et al., 2005). The population dynamics of various species may have been affected in two alternating ways by glaciation: shrinkage during glaciation, followed by expansion from refugia during postglacial recolonization, and survival of a few populations in situ (NEWTON et al., 1999). If all extant populations originated from a single refugium, it seems likely that there would be little among-population differentiation (NEWTON et al., 1999). This might account for the low levels of among-population genetic differentiation reported for most species in the Cupressaceae (JIMÉNEZ et al., 2003; WANG et al., 2004; ALLNUTT et al., 2003; COTTRELL et al., 2005; ZHANG et al., 2005). However, if the species survived in multiple refugia survived during glacial stages, the isolated populations probably experienced strong bottlenecks and severe genetic drift, resulting in elevated levels of genetic differentiation among populations (HEWITT, 1996, 2000). Under this second scenario, *C. gigantea* may have survived in situ during the Quaternary due to its long life span. Subsequently, repeated reduction and expansion of the surviving populations during the climatic oscillations may have resulted in the high among-population differentiation detected in our study and resulted in further reductions in the total genetic diversity across all populations.

Conservation and management

Thorough understanding of the extent and patterns of genetic diversity in *C. gigantea* in the QTP is essential

for its conservation and exploitation. It is important to understand where efforts should be focused to effectively manage and conserve populations of this species. Although a local natural protection area has been established for this species, it only includes the Linzhi population, which is the largest relic population (FU et al., 1999). The four populations we sampled outside the national protected area were genetically distinct from each other (Figure 2). According to the RAPD dendrogram, two distinct groups were recovered. The major population separation is due to longitudinal differences of populations (G4-G5 vs G1-G2-G3) and that there is an altitudinal separation between G1 vs G2-G3. Although the results based on the ISSR data failed to recover such two groups, populations G5, G4 and G1 seem to be distinct and G2 and G3 are closely related. The genetic relationships identified for the two markers were overall similar, and the minor difference detected between them probably reflected the difference in the sampled loci and the different evolutionary processes affecting the genomic regions amplified by the different markers. These results suggested that at least four conservation units comprising populations G1, G4, G5, G2 or G3 should be considered. The high differentiation and the inferred limited gene flow among populations also suggest that as many populations as possible should be used in artificial recruitment and re-planting programs in their original habitats. Therefore, additional populations should be used in conservation efforts if the full range of genetic variation within the species is to be maintained. In addition, losses of genetic diversity may reduce the viability and subsequent regeneration potential of this species (FRANKHAM et al., 2002). Efforts to understand the reduction in *C. gigantea* populations need to be coupled with research on the species' reproductive and demographic processes, especially with respect to the effects of different management strategies on the viability of its populations.

Comparison of RAPD and ISSR data

When the levels of variation detected using the RAPD and ISSR markers were compared, the ISSR primers were found to generate more polymorphic bands than the RAPD markers (Table 3). This trend has been repeatedly found in other herb species (e.g. QIAN et al., 2001). Microsatellites (ISSRs) are short tandem repeating DNA sequences whose repeat lengths are only a few base pairs long. These sequences are abundant and widely dispersed throughout the genomes of eukaryotic organisms and the high level of microsatellite polymorphism is due to DNA slippage, accounting for the difference between the ISSR and RAPD markers (WEBER and MAY, 1989). In addition, a distinct difference was detected in the average genetic distance between populations using the different markers. The inconsistencies between RAPD and ISSR patterns may be a result of the different evolutionary processes and selective forces acting on the genomic regions amplified by the two markers (QIAN et al., 2001). However, the total genetic diversity at the species level, and levels of population differentiation found using RAPD markers were similar to those identified using the ISSR markers, although the absolute values differed. The differences we detected

between ISSR and RAPD markers are consistent with those found in studies of other species (NYBOM, 2004). In terms of amplification reproducibility, ISSR markers are more reliable than RAPDs. RAPDs are highly sensitive to PCR reaction conditions (FRITSCH and RIESEBERG, 1996) and a standard procedure must be established and conducted with caution to obtain good quality, reproducible bands. Less caution is required for ISSR markers, which are robust and less sensitive to PCR reaction conditions. The advantages of ISSRs, compared with RAPD markers, coupled with the higher levels of polymorphism they detect, lead us to conclude that ISSRs are superior markers for tree population genetics research.

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