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Regional genetic differentiation in Western Australian sandalwood (*Santalum spicatum*) as revealed by nuclear RFLP analysis

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Abstract Western Australian sandalwood, *Santalum spicatum*, is widespread in the semi-arid and arid regions of Western Australia, and there is some morphological variation suggestive of two ecotypes. The level and structuring of genetic diversity within the species was investigated using anonymous nuclear RFLP loci. *Santalum spicatum* showed moderate levels of genetic diversity compared to other Australian tree species. The northern populations in the arid region showed greater levels of diversity and less population differentiation than the southern populations in the semi-arid region due to differences in the distribution of rare alleles. Equilibrium between drift and gene flow in the northern populations indicated that they have been established for a long period of time with stable conditions conducive to gene flow. In contrast, the southern populations showed a relationship between drift and gene flow indicative of a pattern of fragmentation and isolation where drift has greater effect than gene flow. The different patterns of diversity suggest that the ecotypes in the two regions have been subject to differences in the relative influences of drift and gene flow during their evolutionary history.

Keywords Genetic diversity · RFLP · Sandalwood · Drift-gene flow equilibrium

Introduction

Sandalwood is highly valued for the sequiterpene essential oil contained in the heartwood (Adams et al. 1975), which is mainly used in the perfume industry but is also used for incense and joss stick manufacture and for carving (Rai 1990). The majority of commercial sandalwood is obtained from the Indian sandalwood (*Santalum album*, Santalaceae), which is harvested from natural populations and plantations in India and Asia (Srinivasan et al. 1992). The Western Australian sandalwood [*S. spicatum* (R.Br.) A.DC.] also produces aromatic wood similar to Indian sandalwood (Adams et al. 1975) and is exported from Western Australia to several Asian countries where it is used mainly in the manufacture of joss sticks (Jones 2001). There are several other species of *Santalum* that occur in Australia, but they do not produce sufficient quantity of the sesquiterpenes that make up the sandalwood fragrance to be commercially significant (Shea et al. 1998).

Western Australian sandalwood has a broad distribution throughout the semi-arid (approximately 300–600 mm rainfall) and arid (approximately 150– 300 mm rainfall) areas of Western Australia but is absent from the high rainfall areas in the extreme south-west and the tropical areas in the north. It is a slow-growing root hemiparasite tree (Hewson and George 1984) that commonly occurs with nitrogen-fixing plants such as *Acacia* or *Allocasuarina* that it uses as hosts (Loneragan 1990). Sandalwood occurs across a wide range of environmental conditions and shows morphological variation, suggesting the presence of two ecotypes within the species (Fox and Brand 1993). In the higher rainfall areas in the south, the species exhibits larger leaves and nuts, higher chlorophyll content and more of a tree habit than in the arid areas in the north of the distribution (Fox and Brand 1993), while trees in the arid areas show a higher concentration of oil in the wood (Loneragan 1990). This morphological variation probably reflects adaptation to environmental conditions but may also have some genetic basis. Variation in growth rates among provenances from both regions has been observed in a field trial (Brand et al. 1999), indicating some genetic influence on growth characteristics, and genetic differentiation of two lineages has been observed in cpDNA (Byrne et al. 2003).

Western Australian sandalwood is obtained from natural populations, but the abundance of the species has been reduced due to extensive harvesting combined

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with poor recruitment and slow growth. This has prompted investigation into the feasibility of commercial production of sandalwood from plantations in the agricultural areas of Western Australia (Shea et al. 1998). Domestication and the development of improvement programs are enhanced by a knowledge of the genetic structure of species in order to capture a broad representation of genetic diversity in breeding populations and seed orchards (Moran et al. 2000). The design of sampling strategies that aim to capture a large amount of the genetic resources within species depends on the amount of diversity within populations and the level of genetic differentiation between populations (Brown and Hardner 2000). Appropriate management of natural populations and in situ conservation of genetic resources also requires information on the distribution of genetic diversity (Moran et al. 2000). This study investigated the level and structuring of genetic diversity within *S. spicatum* to provide a basis for the domestication of Western Australian sandalwood for agroforestry. It will also provide information for the conservation of the sandalwood genetic resource and the management of natural populations that are still harvested for sandalwood production. Isozymes have proved unsuitable for the analysis of genetic variation in this species (Brand 1993) therefore nuclear restriction fragment length polymorphism (RFLP) markers were developed to allow an investigation of genetic diversity using co-dominant genetic markers.

Materials and methods

Genomic library construction

A random genomic library was constructed from *S. spicatum* using standard methods (Sambrook et al. 1989). Total genomic DNA from a single sandalwood tree was digested with *Pst*I, treated with Gene Clean (Geneworks) then ligated into a *Pst*I-digested, dephosphorylated pUC19 plasmid. Plasmids were transformed into competent cells of the bacterial strain Dhα101 (GibcoBRL, Gaithersburg, Md.) by heat shocking. Transformed colonies containing recombinant plasmids were selected using ITPG/X-gal screening and maintained as glycerol stocks at –80 °C. Probes were prepared by PCR amplification of the insert using M13 universal primers and cleaned by passing through filter tips at 6,000 rpm in a microfuge, followed by ethanol precipitation. To screen for a high-copy DNA sequence, plasmid inserts were separated on 0.8% agarose gels, Southern blotted and hybridized with total genomic DNA. Inserts containing a high-copy sequence were discarded. Probe evaluation was carried out by screening 103 probes against DNA from six different individuals digested with each of three restriction enzymes *Bgl*II, *Eco*RI or *Eco*RV. Twenty-eight probe – enzyme combinations that showed scorable single locus patterns (without bias towards polymorphism) were selected to assess the diversity of populations.

Plant material and DNA procedures

Leaf samples were collected from 23 populations across the geographic range of the species in both the southern (semi-arid) and northern (arid) areas (Fig. 1). Samples were also collected from two populations (one southern, one north-eastern) of the related species *S. acuminatum* (Quandong). DNA was extracted from the leaves following the methods outlined in Byrne and Moran (1994) with the addition of 0.1 *M* sodium sulphite to the extraction buffers (Byrne et al. 2001). In comparison to DNA yields obtained from other woody plant species, DNA yields from *S. spicatum* were low, and this may be related to the presence of sesquiterpene oils that interfere with the DNA extraction process. However, sufficient high-quality DNA was obtained from ten individuals for each population. DNA samples were digested with either *Bgl*II, *Eco*RI, or *Eco*RV, Southern-blotted and hybridized with 28 RFLP probes. Hybridization conditions were as in Byrne and Moran (1994).

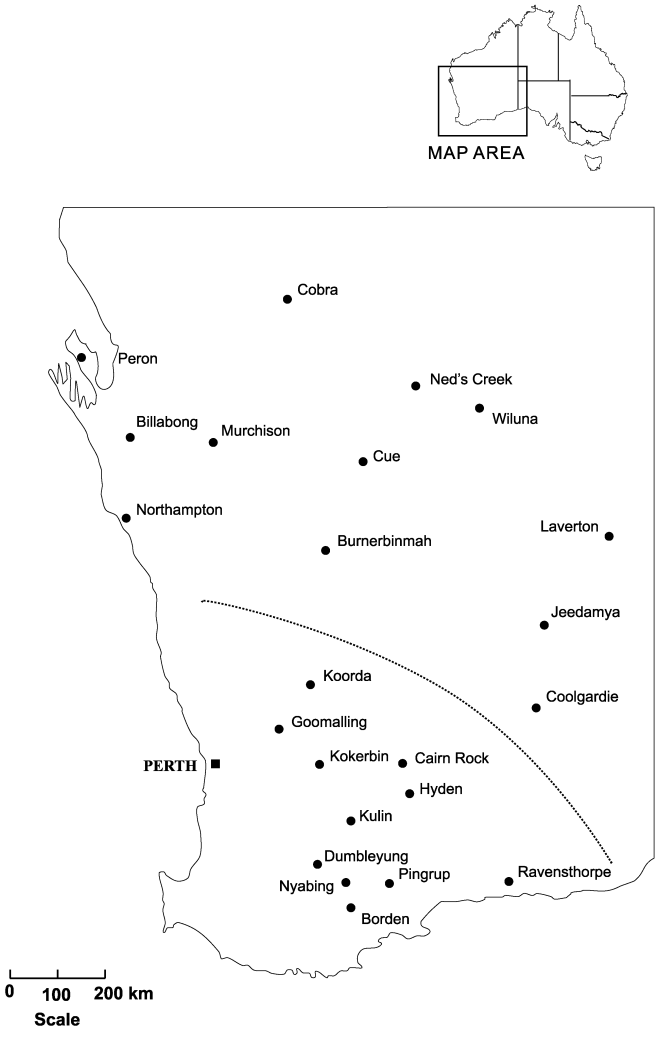

Fig. 1 Location of sampled populations of *Santalum spicatum*. *Dotted line* indicates approximate separation of northern and southern regions

Data analysis

Data analysis was carried out at the species level for all populations and also at a regional level (southern/semi arid region versus northern/arid region) where appropriate. The population at Northampton has a semi-arid, coastal environment but is in the northern area and was included in the northern group of populations for the regional analysis. The banding patterns were interpreted according to a Mendelian multi-allelic model. Allelic diversity parameters and homogeneity tests of allele frequency distributions (G test) were calculated using POPGENE (Yeh et al. 1997). Gene diversity parameters were calculated according to Nei (1978) using

FSTAT (Goudet 2000). Measures of inbreeding (F, f) and population differentiation (θ) were calculated for all populations and for the northern and southern populations separately using the estimates of Weir and Cockerham (1984) which are unbiased for small sample sizes. Confidence intervals (95%) were estimated by bootstrapping over loci 1,000 times.

Regression of pairwise population differentiation (θ) against geographic distance was tested for significance using a Mantel randomization test with 1,000 iterations (Mantel 1967) for all populations and for the northern and southern populations separately. Where a significant result was obtained, the residuals of the regression were also tested against geographic distance to determine whether the degree of scatter also increased with geographic distance as expected under a hypothesis of drift-gene flow equilibrium (Hutchinson and Templeton 1999). Genetic patterns between populations were investigated with the multi-dimensional scaling routine in PRIMER (Clarke and Gorley 2001) using Nei's genetic distance (Nei 1978) as the association measure.

The phylogenetic relationship between populations was determined based on gene frequency using a continuous character maximum likelihood analysis in PHYLIP (Felsenstein 1993). The intention was to use the related species *S. acuminatum* as an outgroup in this analysis, but both populations showed multiple bands that were not consistent with a standard diploid allelic pattern. This was not due to degradation or poor quality DNA since the same filters have been used for a chloroplast DNA study with no problems in interpretation. The nuclear banding patterns obtained here suggest that *S. acuminatum* is polyploid, and it was not included in the data analysis. Therefore, the southern population of Nyabing was specified as the outgroup for the phylogenetic analysis as it was one of three populations that had the greatest average genetic distance from the rest of the populations. The significance of nodes in the dendrogram was assessed by bootstrapping with 100 replications.

Results

Information derived from 27 probes was used in the analysis, since one probe produced a banding pattern that could not be reliably scored. Polymorphism in *S. spicatum* was high, with only one locus monomorphic in all populations. There were no loci that were polymorphic in all populations. The number of alleles detected at a locus ranged from one to ten with the maximum number of alleles in any one population being six. The distribution of alleles in frequency classes showed that the majority of alleles were common with a frequency greater than 0.5 (mean of all populations = 51%) and that the proportion of rare alleles of a frequency less than 0.1 was much lower (mean of all populations = 17%). Nearly half of the rare alleles (46%) were unique (i.e. present in only one population), and there were more rare alleles present in the northern region (31 in 12 populations) than the southern region (16 in 11 populations). The two regions showed some differences in the distribution of alleles within the region, with the northern region having a lower frequency of common alleles and a higher frequency of rare alleles (>0.5 = 47%, <0.1 = 19%) than the southern region (>0.5 = 54%, <0.1 = 12%). In the northern region nearly half of the rare alleles were unique (47%), while the southern region had a lower frequency of unique alleles (38%).

Homogeneity tests of allele frequencies identified significant ($P < 0.01$) differences across populations for a large number of loci in both the northern (13 out of 27 loci) and southern regions (10 out of 27 loci). Inspection of allele frequencies among populations within regions showed that the cause of the heterogeneity was different in the two regions. In the northern region, most loci showed differences between populations in the distribution of rare alleles. However, in the southern region, the differences between populations were due to large differences in the frequency of the common allele and, correspondingly, large increases in the frequency of one or more rare or low frequency alleles, often only in one or two populations. For example, at locus 80, the frequency of allele 3 ranged from 0.6 to 1.0, and allele 1 from 0.000 to 0.350 except at Nyabing where the frequency of allele 3 was 0.333 and allele 1 was 0.667; for locus 95, the frequency of allele 2 ranged from 0.000 to 0.150 and allele 5 from 0.8 to 1.000 except in the Ravensthorpe population where the frequencies were 0.4 and 0.6, respectively. At the species level, homogeneity tests were also significant ($P < 0.1$) for a large number of loci (11 out of 27), but this was often due to the effects of allele frequency differences in either or both of the regions. There were only four loci where allele frequency differences were evident between the regions (Table 1).

Measures of allelic diversity among populations of *S. spicatum* showed moderate levels of diversity for a woody perennial. The number of alleles per locus (A), observed heterozygosity (H_o) and heterozygosity expected under Hardy-Weinberg equilibrium (He) were generally lower in the southern populations than in the northern populations. There were two populations, Goomalling and Borden (Table 2), where the lower diversity was significant. The Borden population is very small and is the southern-most population at the edge of the species range. The inbreeding coefficient, F_{IS}, was generally not significantly different from zero, and the negative values may reflect bias due to small sample sizes. The mean estimates of total genetic diversity showed moderate levels of diversity ($H_T = 0.233$), with the majority of this diversity within populations, although some diversity was maintained between populations ($G_{ST} = 8.3\%$).

Although the majority of variation (92%) occurred within populations, the level of population differentiation at the species level ($\theta = 0.087$) was significantly different from zero based on 95% confidence intervals (Table 3).

Table 1 Frequencies of alleles at four RFLP loci showing the greatest differences between the northern and southern regions in *Santalum spicatum*

Area	s47-2	s47-6	s77-2	s77-3	s64-7	s86-1	s86-5
All populations	0.636	0.277	0.793	0.232	0.709	0.826	0.129
Northern region	0.744	0.182	0.833	0.155	0.762	0.709	0.218
Southern region	0.525	0.378	0.751	0.312	0.656	0.950	0.045

Table 2 Allelic diversity parameters for populations of *S. spicatum*

Population	P[a]	A[b]	H_o[c]	H_e[d]	F_{IS}[e]
Cobra	70	2.2	0.236 (0.018)[f]	0.247 (0.017)	–0.041 (0.027)
Peron	67	2.1	0.230 (0.017)	0.234 (0.017)	–0.087 (0.029)
Ned's Greek	70	2.6	0.226 (0.017)	0.230 (0.017)	–0.050 (0.028)
Wiluna	75	2.0	0.180 (0.015)	0.206 (0.017)	0.005 (0.027)
Murchison River	70	2.0	0.197 (0.015)	0.212 (0.016)	–0.031 (0.027)
Billabong	56	1.9	0.185 (0.018)	0.171 (0.017)	–0.114 (0.029)
Cue	67	1.9	0.221 (0.017)	0.205 (0.017)	–0.106 (0.024)
Laverton	67	2.1	0.278 (0.020)	0.270 (0.018)	0.021 (0.042)
Northampton	67	2.0	0.222 (0.019)	0.223 (0.017)	–0.010 (0.036)
Burnerbinmah	70	2.0	0.160 (0.014)	0.187 (0.016)	0.007 (0.027)
Jeedamya	70	2.3	0.237 (0.017)	0.232 (0.017)	–0.086 (0.025)
Coolgardie	70	1.9	0.224 (0.017)	0.228 (0.017)	–0.003 (0.030)
Mean – northern	68	2.1	0.216 (0.009)	0.220 (0.007)	–0.041 (0.014)
Koorda	70	2.1	0.237 (0.017)	0.243 (0.017)	–0.039 (0.025)
Goomalling	48	1.6	0.126 (0.016)	0.152 (0.016)	0.084 (0.050)
Cairn Rock	63	1.9	0.167 (0.016)	0.188 (0.016)	0.008 (0.038)
Kokerbin	67	1.8	0.174 (0.016)	0.200 (0.016)	0.061 (0.030)
Hyden	60	1.7	0.217 (0.018)	0.209 (0.017)	–0.102 (0.023)
Kulin	63	2.0	0.195 (0.016)	0.216 (0.016)	0.017 (0.036)
Dumbleyung	63	1.8	0.157 (0.015)	0.193 (0.016)	0.071 (0.035)
Pingrup	52	1.7	0.191 (0.018)	0.198 (0.017)	–0.052 (0.045)
Nyabing	63	1.9	0.225 (0.019)	0.228 (0.018)	–0.076 (0.032)
Ravensthorpe	63	2.0	0.235 (0.020)	0.243 (0.019)	–0.021 (0.033)
Borden	30	1.4	0.104 (0.017)	0.114 (0.016)	–0.042 (0.084)
Mean – southern	58	1.8	0.184 (0.013)	0.198 (0.012)	–0.008 (0.018)
Mean – total	63.5	1.95	0.201 (0.009)	0.210 (0.008)	–0.026 (0.011)

[a] P, Percentage of loci that are polymorphic (0.99 criterion)
[b] A, Mean number of alleles per locus
[c] H_o, Observed heterozygosity
[d] H_e, Hardy-Weinberg expected panmictic heterozygosity
[e] F_{IS}, Mean Fixation index over all loci
[f] Standard error in parenthesis

Table 3 Measures of inbreeding in *S. spicatum* calculated using Weir and Cockerham's (1984) estimates

	F[a]	θ[b]	f[c]
Northern populations	0.076 (–0.003, 0.166)[d]	0.055 (0.038, 0.074)	0.023 (–0.055, 0.116)
Southern populations	0.190 (0.109, 0.280)	0.108 (0.079, 0.140)	0.092 (–0.003, 0.199)
Overall	0.137 (0.063, 0.220)	0.087 (0.068, 0.108)	0.055 (–0.024, 0.152)

[a] F, Overall inbreeding coefficient
[b] θ, Coancestry coefficient
[c] f, Degree of inbreeding within populations
[d] Confidence intervals (95%) in parenthesis

The level of population differentiation was higher in the southern region than in the northern region and was also higher than across the species overall. There was no significant association between population differentiation (θ) and geographic distance across the whole species as assessed by a Mantel test (g = 1.213, P = 0.131). There was also no significant association for the populations in the south (g = 0.276, P = 0.379); however, there was a significant association for populations in the north (g = 2.167, P = 0.021). For the northern populations, the residuals from the regression also showed a highly significant association with geographic distance (g = 7.546, P = 0.001).

Unbiased estimates of genetic distance between all pairwise comparisons of *S. spicatum* populations were low, with the highest distance (0.073) occurring between the southern populations at Nyabing and Pingrup. The lowest genetic distance (0.006) occurred between the northern populations of Ned's Creek and Cue. The average genetic distance between populations across the whole species was low (0.042). Average genetic distance between southern populations was similar to that across the whole species (0.044), but the average distance between northern populations was lower (0.031). The MDS ordination plotted in three dimensions produced a stress value of 0.12, indicating a fair fit between the ordination and the data. In three dimensions the ordination showed a clear gradient from the southern to northern populations with no overlap between the two groups. The ordination in two dimensions is shown in Fig. 2. Populations from the two regions occupied separate ordination space except for the northern population at Laverton that was within the group of southern populations. The spatial

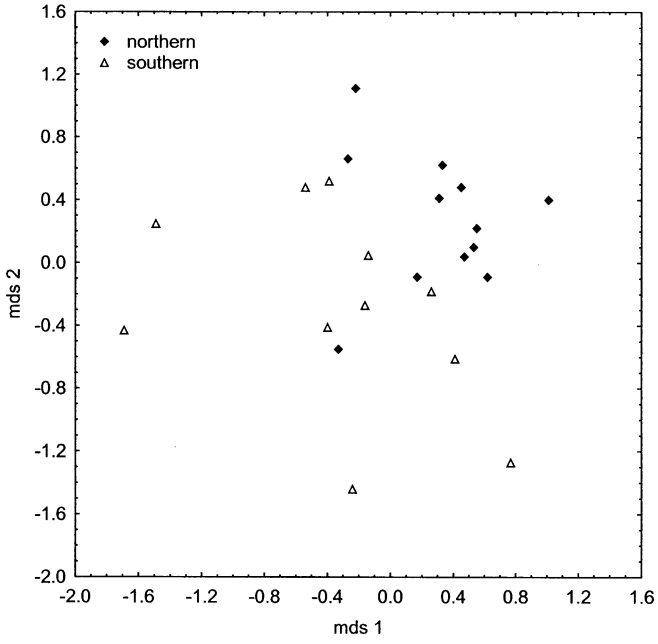

Fig. 2 Ordination analysis in two dimensions of genetic distance between populations of *S. spicatum*. *Black diamond* Northern populations, *White triangle* southern populations

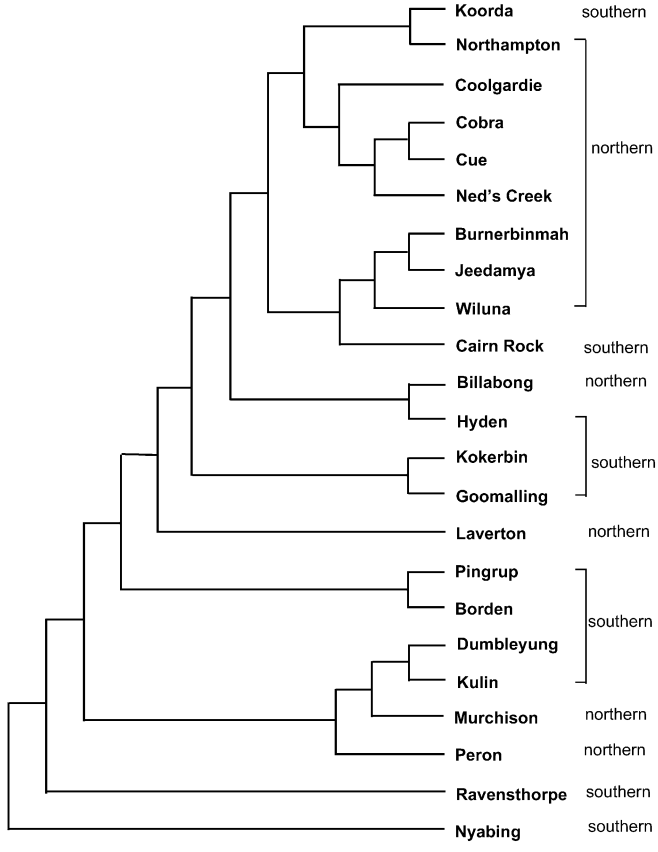

Fig. 3 Consensus tree for population phylogeny (continuous character maximum likelihood) analysis of populations of *S. spicatum*

separation of the southern populations was greater than that of the northern populations.

A population phylogeny based on maximum likelihood analysis showed some evidence of genetic structuring within *S. spicatum* (Fig. 3), with the northern populations having a greater degree of association than the southern populations. The majority of northern populations were grouped into a clade, although two southern populations were also clustered in this clade. Some pairs of wheatbelt populations showed similarities, but the majority of populations were divergent and showed little association. None of the nodes were significant with all bootstrap values less than 40% (not shown).

Discussion

Many widespread tree species exhibit patterns of genetic structuring between geographic regions (Moran 1992), particularly where there are barriers to dispersal between the regions such as mountains, valleys or oceans. Differences in regional history resulting in variable influences of evolutionary forces can also lead to different patterns of genetic structure between regions (Hutchinson and Templeton 1999). Analysis of genetic diversity in the widespread *S. spicatum*, which covers both semi-arid and arid environments, has shown differences in population structure between these regions. Populations in the north showed higher levels of genetic diversity and a greater number of rare alleles than those in the south. Populations in the south had almost double the level of differentiation between populations than those in the north and also showed a greater level of average genetic distance between populations. These genetic differences are related to the different patterns of allele frequency distribution between populations in the two regions. In the northern region, the pattern of differentiation appears to be due to numerous rare alleles that are present at a low frequency in one or a few populations. In comparison, the populations in the southern regions had fewer rare alleles, and while the majority of these were shared by several populations, the frequency of these alleles was often very much higher at one or more populations in conjunction with a lower frequency of the common allele at the locus. These different patterns of allele frequencies lead to different levels of differentiation between populations in the two regions.

The degree of differentiation between populations is related to the interaction and relative influences of both drift and gene flow, with drift increasing differentiation of populations and gene flow decreasing differentiation. Gene flow though pollen dispersal would be expected to be low in *S. spicatum* as it is insect-pollinated. Seed dispersal may be moderate as the seeds are large nuts which can be eaten by emus (*Dromaius novaehollandiae*), and the woylie (*Bettongia penicillata*), a small mammal, is hypothesized to have cached seeds, which would also be a means of seed dispersal (Havel 1993). The reproductive biology of the species is the same in both

ecotypes so gene flow would be expected to be similar in both regions, although the abundance of emus and woylies would have been greater in the southern region than in the northern region. However, higher seed dispersal in the southern region is not consistent with the higher level of differentiation observed in the southern region. The populations in the south are geographically closer than those in the north, so drift would be expected to be stronger in the north due to greater geographic isolation. However, this is also not consistent with the lower level of differentiation observed in the northern region. Therefore, simple effects of recent drift and gene flow do not explain the observed pattern of differentiation between the regions.

Current patterns of differentiation may also be significantly influenced by historical patterns of drift and gene flow. Populations that have existed in suitable conditions for a long enough period of time may reach an equilibrium between gene flow and drift such that differentiation is inversely proportional to gene flow (Hutchinson and Templeton 1999). Species where population structure has been influenced by recent historical events, such as range expansion since glaciation, will not be expected to show drift-gene flow equilibrium (Schaal and Olsen 2000). However, species occupying ancient landscapes that have had a stable evolutionary history, such as that in south-western Australia, may be expected to be in drift-gene flow equilibrium, and isolation by distance, suggesting equilibrium, has been shown in several species in this area (Coates 2000).

The northern populations of *S. spicatum* exhibited a significant association between differentiation and geographic distance that is indicative of equilibrium between drift and gene flow, whereas the southern populations did not show equilibrium. The detection of drift-gene flow equilibrium in the northern region implies that the population structure in this region has been stable for a long period of time. In contrast, a lack of equilibrium in the southern region suggests that either it has not been occupied for sufficient time to allow equilibrium to be reached or that stable conditions have not been present throughout the history of the region. The first scenario would suggest that the southern populations represent a later expansion of the species from longer established populations in the northern region, although the expansion would have been sufficiently long ago for differentiation between populations to develop. The second scenario is consistent with proposals of climatic instability from cyclic expansion and contraction of the mesic and arid zones during the Pleistocene, leading to fragmentation and isolation in the intermediate areas (Hopper et al. 1996). The southern region is between the mesic and arid zones of south-west Western Australia in the area where the greatest influences of climatic instability have been predicted and therefore would have experienced reduced gene flow due to historical fragmentation and isolation. The variance in estimates of divergence for the southern region showed a wide degree of scatter independent of geographic distance that also indicates that drift has been more influential than gene flow, and this would arise if the species had been fragmented into small isolated populations (Hutchinson and Templeton 1999). In contrast, the northern region would not have experienced such climatic instability and would have maintained suitable conditions for gene flow. The pattern of large differences in allele frequencies between southern populations is also consistent with drift being most influential, while the similarity of common allele frequencies and presence of rare alleles in the northern populations suggests gene flow has been effective but mutation is also generating new variation. Thus, historical influences of fragmentation and isolation appear to be the best explanation for differences in the observed patterns of regional genetic diversity in *S. spicatum*.

At the species level, the differences in regional genetic diversity result in a moderate, but significant, level of differentiation between populations and a separation of the regions in the ordination analysis. The regional differences were not as clear in the phylogenetic relationships between populations although some evidence of the regional structure was present in the dendrogram. In comparison with other tree species where genetic diversity has also been assessed using RFLP loci, the level of genetic diversity in *S. spicatum* is lower (H_T = 0.233) than that reported for *Eucalyptus kochii* (H_T = 0.514; Byrne 1999) and *E. loxophleba* (H_T = 0.418; Hines and Byrne 2001). These species are also widespread in the south west of Western Australia, although they do not occur in the arid region. The lower level of gene diversity in *S. spicatum* compared to eucalypts is probably related to the distribution of allele frequency. *Santalum spicatum* had a low levels of rare alleles (17%) and a high level of common alleles (51%) compared to the eucalypt species where the frequency of alleles is skewed towards rare alleles (33–66% rare alleles, 11–22% common alleles). *Santalum spicatum* also maintained a higher proportion of diversity (G_{ST} 8.3%) between populations than the two eucalypts (2.2 and 3.8%).

The regional differences in genetic structure associated with differences in the relative influences of evolutionary processes in *S. spicatum* is congruent with the presence of different chloroplast DNA lineages in the regions (Byrne et al. 2003) and consistent with the identification of northern and southern ecotypes based on morphological variation (Fox and Brand 1993). The pattern of differentiation in the southern region indicates that germplasm collection strategies should sample across the range of populations to ensure adequate representation of the variation. It also suggests that there may be variation in quantitative traits for which genetic gain could be achieved by a program of selection and breeding for sandalwood in this region. Regeneration of natural populations through seedling establishment is the main management issue in the northern arid region. The genetic pattern in this region suggests that seed used in regeneration should be sourced from local areas so as not to disturb the balance between drift and gene flow that has developed in this region.

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