**Patterns of morphological and molecular variation in *Conospermum triplinervium*
R.Br (Proteaceae), and their implication for bushland restoration.**

Elizabeth Sinclair^{A, B, D}, Belinda Cheetham^{A, B}, Siegfried Krauss^{B, C}, Richard Hobbs^A

^AEnvironmental Science, Murdoch University, Murdoch, WA 6150 Australia

^BBotanic Gardens and Parks Authority, Fraser Avenue, West Perth, WA 6005 Australia

^CSchool of Plant Biology, University of Western Australia, Stirling Highway, Crawley,
WA 6009, Australia

^DCorresponding author. Email: esinclair@iinet.net.au

Running head. Patterns of variation in *C. triplinervium*

Abstract. Ecological restoration benefits from information on population genetic structure and variation within a species to make informed decisions on where to source material of the local genetic provenance. *Conospermum triplinervium* is extremely rare in Bold Park, a large bushland remnant currently undergoing restoration in Perth, Western Australia. We sampled plants from Bold Park and six other native populations across the northern half of the species' range to assess patterns of morphological and genetic variation. There was considerable variation across six leaf measures with significant differentiation among some populations. The molecular data showed a high level of population structure ($\Theta^B = 0.4974$), with varying degrees of spatial overlap among populations in an ordination plot. Significant differentiation was observed among all pairs of populations, except for Bold Park and its geographically closest populations at Kings Park and Neerabup. These two populations had greater genetic variation (50.9 and 54.5% polymorphic markers, respectively), compared to 20.2% at Bold Park. The small Bold Park population would benefit from augmentation (via cuttings) from local plants. However, in the longer term, should the Bold Park population show evidence of declining viability then material should be sourced from the genetically similar Kings Park population to increase genetic variation whilst maintaining genetic integrity.

Conospermum triplinervium R.Br

Introduction

Developing strategies for conservation of biodiversity, particularly in species rich 'hotspots' (Myers *et al.* 2000; Cowling *et al.* 2003) continues to be of major concern, due to increasing urbanisation, fragmentation, and climate change (Davis and Shaw 2001; Jump and Peñuelas 2006). Ecological restoration practitioners and conservation biologists also need to recognize the considerable intraspecific diversity, which represents the potential of a species to adapt in a changing environment (van Andel 1998; Newton *et al.* 1999). This can have a significant effect on the outcome of restoration programs (van Andel 1998). In the long term, the level of intraspecific variation may play a decisive role in whether a population persists (Lande and Shannon 1996) and should therefore be considered if restoration and conservation actions are to be successful.

Unprecedented rates of climate change, coupled with land use changes impeding natural gene flow, can be expected to disrupt the interplay between adaptation and migration, likely affecting productivity and threatening the persistence of many species (Davis and Shaw 2001). In the Southwest Australian floristic region (Hopper and Gioia 2004), habitat fragmentation, land degradation, and urbanisation have played key roles in the decline and loss of flora and fauna. Habitat fragmentation and now severe degradation of remaining bushland mean that effective plant conservation requires ongoing and intensive restoration efforts. Restoration projects often require sourcing plant material off-site, whether they are urban bushland remnants or larger scale landscape restoration projects. Information on population genetic structure of key restoration species can contribute to the decision-making process for sourcing appropriate seed (or green stock).

Conospermum triplinervium R.Br. (Proteaceae), the tree smokebush, has a disjunct distribution throughout its range from Zuytdorp National Park in the north to Albany in the southwest, with a more continuous distribution between Eneabba and Yalgorup (Fig.1). It is rare in the far southwest part of its range (Bennett 1995), and experiencing considerable decline within the Swan Coastal Plain due to urbanisation and degradation of urban bushland remnants. There are, however, several significant populations within

the Perth metropolitan area designated as Bushland Forever sites (Dell and Banyard 2000). The species is of concern in Bold Park, a 437 ha bushland remnant undergoing restoration in the western suburbs of Perth, as there are only four plants remaining – one senescing and three new plants that have germinated since a fire in 2000. A key management objective is that no species are to be lost from Bold Park (BGPA 2000). Although *C. triplinervium* readily germinates after disturbance, the frequency of fires in the park (10 in last 40 years; J. Fisher pers. comm.) has meant that the soil seed bank has probably been depleted and it is unlikely to recover naturally. The nearest population is in Kings Park, 5.6km to the east.

There has been some confusion in the taxonomy of *Conospermum* spp., however, the descriptions of *C. triplinervium* (single stemmed tree) and *C. canaliculatum* (multi-stemmed shrub) are quite clear in the most recent revision of the genus (Bennett 1995). *Conospermum triplinervium* is a small tree to 4m, mostly lacking a lignotuber and killed by fire, but readily regenerates from seed (Bowen 1991; Bennett 1995). There is considerable variation in leaf size and shape, and the broad leaf form of *C. canaliculatum* intergrades with the narrow-leafed form of *C. triplinervium* (Bennett 1995). Several species are largely sympatric with *C. triplinervium* across the Perth metropolitan area including *C. canaliculatum*, *C. stoechadis*, and *C. undulatum*, and complex hybridisation event(s) may occur (G. Messina, pers. comm.).

The focus of this research is on the ecological restoration of Bold Park and other urban bushland remnants, as part of a larger study to develop a genetic provenance atlas for the Swan Coastal Plain (Bussell *et al.* 2006; Krauss and He 2006). A critical question in restoration involves the source of genetic material with respect to local adaptation and the maintenance of natural patterns of genetic structure (Krauss and He 2006; Montalvo *et al.* 1997). The assessment and contrast of patterns of both morphological and genetic variation contributes to an understanding of local adaptation and gene flow within a species (eg Broadhurst *et al.* 2006). Here, we examine morphological (leaf and inflorescence) and genetic (Amplified Fragment Polymorphic DNA; AFLP) variation to understand population structure across the more continuous range of *C. triplinervium*, to

help identify genetically integrated source population(s) for the restoration of Bold Park. We also investigate whether low seed set and/or viability may be a contributing factor for the current low plant numbers in Bold Park.

Materials and Methods

Sampling

The distribution of *C. triplinervium* falls into three to four geographic groups, which may represent different taxa (R. Barrett, pers. comm.). As a consequence, our sampling included only those populations from the mostly continuous part of the range between Eneabba and Perth (Fig. 1). We were unable to locate any populations from Florabase records between the Swan River and Yalgorup National Park (along the coast, south of Perth). We sampled from 'pure' *C. triplinervium* populations (single-stemmed, tree-form only). Fresh leaf material was collected from seven locations between December 2005 and April 2006 (Fig. 1). All populations were in the Spearwood and Bassendean dune systems of the Swan Coastal Plain, with the exception of Julimar Forest, which was in a winter wet depression on the western edge of the Darling Scarp. Sites were generally selected if they contained at least 20 plants. Collections from Bold Park, Kings Park, and Julimar Forest included all or most of the known plants. The populations at Welshpool, Neerabup, and Nambung Flats form almost complete monocultures of similar aged plants, while plants in Julimar Forest and parts of Kings Park were largely senescing. Leaf shape varies with age in *C. triplinervium*. Juvenile leaves are broad while mature leaves are more elongate, although there is considerable variation in mature leaves (Fig. 2). Five mature leaves were collected from each plant for leaf phenology, taking leaves from different branches where possible and avoiding damaged leaves. These were kept in the refrigerator until scanned. A small branch containing several leaves was collected onto ice in the field and then stored at -80°C until required for DNA extraction. Five inflorescences were also collected per plant (if flowering) to estimate seed set and viability. Additional material was collected and vouchered from each site (Table 1). Vouchers were deposited in the State Herbarium of Western Australia.

Phenology

Leaves were scanned at 300 dpi as soon as possible after collection, five at a time (from a single plant), using CanoScan Toolbox 4.9. The images were processed using 'Image Tool' software (developed at the University of Texas Health science Center, San Antonio). By adjusting the background contrast, the software was able to recognize each leaf image. Six parameters were measured for each leaf including total leaf area (mm²), perimeter (mm), length of the major axis (length, mm), length of the minor axis (width, mm), elongation (ratio of the length of the major axis to length of the minor axis), and roundness ($4 \times \text{perimeter} \times \text{total area} / \text{perimeter}^2$).

One-way analysis of variance (ANOVA) was used to test for differences between sampled populations and each leaf measurement using SPSS (2000). When a significant F-value was obtained in an ANOVA, Scheffe's F procedure for post hoc comparisons was used to determine which population means were significantly different for each of the six measurements in Excel. A Bonferroni correction was used to adjust for multiple comparisons, where the new alpha was set at α/N comparisons. We used the average of five leaves per plant so the data sets were comparable to the AFLP dataset (one data point per plant). Non-metric multidimensional scaling (MDS) was used to visualise variation across the populations for the six leaf measure variables using Primer v5 (Clarke and Gorley 2001). The elongation data were log transformed, using $\log(0.1+V)$ where V = value, as they did not fit a normal curve. A normalised Euclidean distance matrix was generated and 100 restart replicates were run from a random start.

Inflorescences

Conospermum triplinervium is known for producing large numbers of inflorescences, so we limited sampling to five inflorescences per plant. Inflorescences were collected randomly from each plant. Four measures were taken: length of the inflorescence, number of terminal branches; number of flowers per terminal branch (as measured by the presence of a flower or scar as flowers readily drop off during handling), and number of fruit (containing a single seed; present or indicated by an enlarged bract). An average of

each of the four measures was calculated for each population. A ratio between number of flowers and number of seeds set was also calculated per population to obtain a value for the proportion of seeds set. Seeds were collected from all inflorescences, combined by population, and subjected to X-rays (20kV for 10 seconds) generated using very low voltages (Faxitron Xray Corporation) to determine the proportion of viable seeds. Seeds were recorded as viable when a full embryo was observed, non-viable if an embryo was not observed or had shrunk considerably in size, and undetermined if it was not possible to determine (Fig. 3). We restricted these analyses to inflorescences collected from Bold Park, Kings Park, and Welshpool populations, as they were collected at the same time of year (December 2005). We also included fresh seed collections from Kings Park (November 2006) to determine if there was a difference in seed viability after 12 months storage at room temperature.

Molecular Markers

Genomic DNA was extracted from fresh frozen mature leaves using a Qiagen kit (Qiagen Inc.), with approximately 0.250g of leaf ground in liquid nitrogen prior to extraction. AFLP involved three steps, restriction-digestion, pre-selective PCR amplification, and selective PCR amplification. Restriction of genomic DNA was done at 37° C for 2 hr in a 20µl volume containing approximately 250ng of DNA, 2.5U of *Mse*I and 5.2U *Pst*I, 2.0µl NE buffer 2 (supplied with *Mse*I enzyme), 2.0µl 0.1% BSA, and DNA-free water. Next, 5µl of a solution containing 4.0µl *Mse*I/*Pst*I-adapters, 0.5µl T4 ligase, and 0.5µl ligation buffer (supplied with T4 ligase) was added to the samples and further incubated at 20° C overnight, then diluted 1/20 in DNA-free water. Pre-selective PCR was performed in a 20µl total volume containing 4.0µl 5X PCR buffer containing dNTPs, 1.2µl MgCl₂ (25mM), 0.5µl each of *Pst*I and *Mse*I primers (5µM), 0.825U *Taq* DNA polymerase (Fisher Biotech), 4.0µl restricted/diluted DNA template and DNA-free water. The PCR was performed in a PerkinElmer Applied Biosystems 9700 thermal cycler for 20 cycles each at 94° C for 30s, 56° C for 2 min, 72° C for 2 min. A final extension step at 72° C for 5 min was performed. PCR products were diluted 1/30 with DNA-free water for subsequent, selective amplification. Selective PCR was done in a 10µl total volume

containing; 2.0µl 5X PCR buffer containing dNTPs, 0.6µl MgCl₂ (25mM), 0.25µl fluorescently-labelled *Pst*I primer (1µM), 0.5µl *Mse*I primer (5µM) (Sigma GenoSys), 0.25U *Taq* DNA polymerase, 2.5µl of diluted pre-selective PCR product, and DNA-free water. The selective PCR cycle consisted of a touchdown cycle for 13 cycles at 94° C for 30s, 65-53° C for 30s, 72° C for 1 min, followed by 25 cycles at 94° C for 30s, 56° C for 2 min, 72° C for 2 min, and a final extension at 72° C for 2 min. *Pst*I and *Mse*I primers sequences are from Muluvi *et al.* (1999). Three primer pair combinations were used; m-CTT/*Pst*-AC (D4 label), m-CTT/*Pst*-CT (D3 label), and m-CTT/*Pst*-CG (D2 label). Selective PCRs were amplified separately, but pooled (D4 - 1µl, D3 - 2µl, and D2 - 4µl) prior to visualizing with a Beckman capillary machine (internal size standard 400). Fragments were scored for the presence (1) or absence (0) of peaks unambiguously between 60 and 400 base pairs. Replicate samples were run on each 96-well plate for consistent scoring of bands.

Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992; Huff *et al.* 1993) was performed using GenAlEx (Peakall and Smouse 2001) to partition genetic variance within and among the sampled populations and regions. Non-metric multidimensional scaling (MDS) was used to visually represent the relative degree of genetic similarity among individuals and the distinction of populations using Primer v5. A Euclidean distance matrix was generated and 100 runs from a random start were made. Pairwise Fisher's exact tests (Raymond and Rousset 1995) were performed to determine which pairs of populations were significantly different from one another using the computer program TFPGA (Tools For Population Genetic Analysis; Miller 1997). We used a Bayesian approach to describe population subdivision, Theta-B (θ^B), an F_{ST} analog for dominant markers (Holsinger *et al.* 2002), using the software Hickory v1.0.4 (<http://darwin.eeb.uconn.edu/hickory/software.html>). Theta-B was estimated under three different statistical models: $f=0$ (no inbreeding), $\theta^B=0$ (no subdivision), and a Full model. Deviance Information Criterion (DIC) are used to select the best-fit model in Bayesian methods, combining a measure of model fit ($\bar{D}$) and model complexity (pD); pD is the effective number of parameters, and it is calculated as $\bar{D} - \hat{D}_{\text{hat}}$, where $\hat{D}_{\text{hat}}$

is -2 times the log likelihood at the posterior mean (see Holsinger and Wallace 2004). The preferred model is one that minimizes $D_{hat} + pD$. As recommended by Rousset (1997), isolation by distance was tested using the correlation between $\theta^B/1 - \theta^B$ and geographic distance ($\log_{10}$ transformed), via a Mantel test (999 iterations) in GenAlEx. Associations between genetic diversity (percentage polymorphic loci, PPL), latitude, longitude, and climatic variables (Table 1, obtained from the Bureau of Meteorology website <http://www.bom.gov.au>) were tested using Pearson's correlation coefficients using Statistica software (StatSoft 1995).

Results

Phenology

Population means and standard errors are given for each measure (Table 2), with an ANOVA indicating significant population differentiation ($F_{crit} = 2.22$, P-value < 0.0001). Scheffe's post-hoc tests identified those populations that were significantly different from each other by leaf shape character (significant values only shown in Table 3). Julimar Forest accounted for 13 out of 16 significant pairwise results. Leaves from Julimar Forest were much smaller in area and more elongated. With the exception of several plants, the MDS grouped all plants into a single cluster, with each population exhibiting considerable variation (Fig. 4a). The stress was low ($=0.09$) suggesting a good representation of the data with a low prospect of misinterpretation. Kings Park, Julimar Forest, and Nambung Flats were largely non-overlapping with each other (and accounted for most of the significant post-hoc tests), while Bold Park, Yanchep, Welshpool, and Neerabup overlapped extensively.

Inflorescences

Bold Park plants produced the longest inflorescences, with the highest number of terminal branches and average number of flowers (Table 4). The ratio of flowers:seeds was similar across the three sites, although Bold Park had a slightly lower percentage seed set. Welshpool and Bold Park had the highest proportion of viable seeds in 2005 (60% and 72%, respectively). The proportion of viable seeds from Kings Park in 2006 was higher than in 2005. Insect borer holes were observed in all three populations collected in 2005 (3.33 - 4.66%). All seeds with borer holes were non-viable, with the exception of one that was undetermined. The most significant difference was in the total number of seeds produced by plants in the Welshpool population (averaging 45 seeds per inflorescence, relative to 18 and 15 for Kings Park and Bold Park, respectively).

Genetic diversity

228 bands were scored for 143 *C. triplinervium* plants; m-CTT/*Pst*-AC (94 bands), m-CTT/*Pst*-CT (71 bands), and m-CTT /*Pst*-CG (63 bands). Overall, 95.6% of AFLP bands were polymorphic across all individuals (Table 5). The highest amount of diversity was observed in the Nambung Flats population (61.4% markers polymorphic) and the lowest was observed in Bold Park (20.2% markers polymorphic). The Welshpool site also had a lower level of variation (37.7% markers polymorphic), with fewer bands present in the population. AMOVA partitioned 65% of the genetic variation to within relative to among (35%) populations ($p = 0.001$). The MDS separated *C. triplinervium* samples based on sampling location for four of the seven populations (Fig. 4b). This pattern was generated by all three primer pairs (not shown), as well as the combined analysis. Bold Park overlapped extensively with Kings Park and Neerabup, which are its geographically closest populations. Bold Park showed the lowest level of subdivision with Neerabup and Kings Park ($\theta^B = 0.0595$ and 0.1047 , respectively). The Welshpool population was the most divergent population (high pairwise θ^B values, Table 6). All populations were

significantly differentiated from each other (significant exact tests, Table 6), with the exception of Bold Park, which was not significantly differentiated from any of the sampled populations.

The DIC components were lowest for the Full model for each primer pair and the combined data set (Table 7). Overall population subdivision, θ^B (Full model = 0.4974, 95% CI = 0.4731-0.4975), was high. The Full and $f=0$ model had similar Dbar and Dhat components, suggesting that one model was not strongly favoured over the others. The $f=0$ model, however, was strongly favoured over the $\theta^B=0$ model for Dhat and Dbar, indicating differences in allele frequencies among populations. Theta-B was also estimated for an f-free model, as Holsinger and Lewis (2003) suggest there are problems with estimating f from dominant data. The values for the f free model were similar to those for the Full model (not shown). There was no significant relationship between θ^B and geographic distance over all populations (Mantel test, $r^2 = 0.002$, P-value = 0.350), or even with Welshpool excluded (Mantel test, $r^2 = 0.1668$, p = 0.900). There were no significant relationships between genetic diversity, latitude, longitude, and climatic parameters.

Discussion

Population structure

There was a high overall level of genetic diversity (218/228 = 95.6% markers polymorphic) in *C. triplinervium*, although only low to moderate levels of genetic diversity within populations (20.2- 61.4% markers polymorphic). Close *et al.* (2006) report a similar level of AFLP variation in the rare and geographically restricted *C. undulatum* (35/37 = 94.6%). The high overall levels of polymorphism are similar to another Proteaceous species, *Banksia hookeriana* (230/238 = 96.6%, Krauss *et al.* 2006), although all are considerably higher than the rare *Grevillea scapigera* (108/143=75.5%; Krauss *et al.* 2002) or endangered *Grevillea caleyi* (56/127 = 44.1%, Llorens *et al.* 2004).

High levels of diversity are consistent with an outcrossing reproductive system common in the Proteaceae (Goldingay and Carthew 1998). Significant genetic differentiation was detected among all populations of *C. triplinervium*, with the exception of Bold Park. However, the non-significance of differentiation for Bold Park is due to the very small population size reducing statistical power. The Welshpool population was most unique genetically, and contained plants that produced three times as many seeds per inflorescence than Kings Park and Bold Park plants. While there was clearly some population differentiation in leaf characters, with Julimar Forest being the most different, the large amount of variation within populations and age of the population explain most of the variation. No significant relationships were observed between latitude, climatic parameters, and genetic diversity, although genetic diversity decreased with increasing rainfall, perhaps suggesting that selection has influenced the patterns of genetic variation. Geographic and climatic trends in allele frequency have been reported in *Eucalyptus camaldulensis* (Butcher *et al.* 2001).

The most recent revision of the genus *Conospermum* (Bennett 1995) clearly distinguishes between *C. triplinervium* (the only tree form) and *C. canaliculatum* (multi-stemmed shrub), despite similar leaf morphology. This study included only those plants that were growing as single-stem trees to avoid taxonomic issues that may complicate the interpretation of AFLP data. However, a single tree (Ct94) at the Julimar Forest site appears to have an intermediate form – a single-stem tree with narrow, slightly recurved leaves more typical of *C. stoechadis*, but with a triple venation typical of *C. triplinervium*. *Conospermum stoechadis* plants also occurred within 100m of this *C. triplinervium* population. Despite what appears to be a morphological hybrid, this individual clustered within the range of genetic variation observed in the other (*C. triplinervium*) samples from Julimar Forest (Fig. 4b). Hybridisation has been reported between eastern states species *C. taxifolium*, *C. ericifolium*, and *C. ellipticum* that have only partially effective barriers to gene flow (Morrison *et al.* 1994). While this study did not set out to determine the extent of hybridisation among the sympatric *Conospermum* spp., the genetic distinctiveness of the Welshpool population from other *C. triplinervium* populations and *C. undulatum* (Close *et al.* 2006), as well as the intermediate sample

Ct94 from Julimar Forest, suggest further work is required. Houston (1989) described a new species of bee pollinator, *Leioproctus conospermi*, which has been recorded from both *C. triplinervium* and *C. stoechadis* flowers. Further sampling should include more southerly populations of *C. triplinervium*, as well as the other sympatric taxa from the Welshpool area (*C. undulatum*, *C. stoechadis*, and *C. canaliculatum*), to provide more insight into the genetic relationships of *Conospermum* spp. on the Swan Coastal Plain. This unique population at Welshpool is also one of the larger remnant populations within the Perth Metropolitan area, with an estimated population of 800 plants.

Management recommendations

An integrated approach to data collection provides a better understanding of variability in a species (e.g. Broadhurst *et al.* 2006). We recognize that there are limitations including time and ability to germinate and grow a species, therefore translocation/growth trials are not always possible, particularly in longer-lived species. In limiting our geographic sampling range (and including morphological and molecular data) to include the more continuous part of the range between Eneabba and Perth, and only sampling single-stemmed trees, we have been able to identify suitable source material. The identification of a suitable source population in combination with appropriate nursery propagation skills provides a practical restoration solution for the very small Bold Park population of *C. triplinervium*.

The percentage seed set (14.5%) and seed viability (72%) in Bold Park are comparable to the much larger populations in Kings Park and Welshpool, although the total number of seeds produced was considerably higher in Welshpool. Levels of genetic diversity were much lower in Bold Park (20.2% markers polymorphic), a trend also observed in other species (e.g. Krauss and He 2006; Sinclair *et al.* 2006; Sinclair and Hobbs, accepted), possibly reflecting a common evolutionary history from a combination of the physical isolation of the Park, length of time isolated, and small population size. Bold Park was not significantly differentiated, based on leaf morphology or genetic variation, to any other sampled populations in this study, although the small sample size (n=4) limits the power of statistical analyses of the genetic data. The MDS shows Bold

Park is genetically most similar (complete overlap) with its geographically nearest and relatively large population at Kings Park, suggesting this population would make the most suitable source population of those sampled should it be determined that 'genetic rescue' is required. The Kings Park population has considerable genetic diversity (50.9% markers polymorphic) and was not significantly differentiated from Bold Park (Exact test, $P = 1.0000$). Both grow on the same Spearwood Dune system, with sands derived from Tamala limestone.

High predation of seeds was reported in a preliminary trial for *C. undulatum* (Close *et al.* 2006). Preliminary trials in *C. triplinervium* showed 100% removal by ants, indicating that there is a very limited opportunity for seeds to remain in the soil seed bank (G. Messina, pers. comm.). A combination of insect attack on maturing seeds (observation of bore holes in seeds collected and still attached to an inflorescence), low seed set (14.5 - 17.0%) and ant predation after seed release, contribute to the low number of germination events. One hypothesis (Wright 1994) suggests that low and variable seed set (in Proteaceae) could act as a defence mechanism against insect seed predators. While we did not set out to test this hypothesis, there was a considerable difference in seed viability at Kings Park between 2005 and 2006 (see Table 4). *Conospermum triplinervium* is fire sensitive as most plants lack a lignotuber, although some plants were found regenerating from a 1989 fire in Kings Park (Bennett (1995). Given that percentage seed set in the Bold Park population is similar to other sampled populations an alternative explanation for the diminishing Bold Park population may be the fire regime. Unfortunately, survey records for Bold Park do not provide information on the size of the *C. triplinervium* population prior to the 2000 fire that burned through one of two locations where *C. triplinervium* naturally occurs in the Park.

The high ant predation on seeds, requirement for disturbance (fire or mechanical), and extremely low germination rates (1/285 seeds under nursery conditions, A. Shade, pers. comm.), suggest seed broadcasting or collection of seed for germination are not appropriate for any restoration actions. Propagation through cuttings is a feasible alternative (eg. Perry and Trueman 1999), although the Western Australian species are

considered difficult to propagate. *Conospermum triplinervium* (var *triplinervium* from Kalbarri) is considered the easiest *Conospermum* spp. to grow in cultivation and produces up to 60% viable seeds (Sainsbury 1991), which is comparable to what we observed in wild populations. The BGPA nursery has been propagating *C. triplinervium* from cuttings collected in Kings Park since 1999, (725/2709, 26.7% successful) and Bold Park in 2002 and 2004 (29/120 = 24.2% successful; A. Shade, pers. comm.). In a preliminary trial using 100 cuttings from the Welshpool population, 24.0% grew (G. Messina, pers. comm.). Despite this low success rate, propagation from cuttings remains the best way to augment the Bold Park population. Since propagation from cuttings is a form of clonal reproduction, cuttings should be taken from as many plants as possible to maximize genetic variation in the new plants. Plants do not require additional watering or tree guards for survival once planted (S. Easton, pers. comm.). Despite the small population size and lower level of genetic diversity relative to the other sampled populations, there was no evidence of a negative impact on reproduction (% seed set) or inbreeding depression (% viable seeds). A recent check (late 2007) of the Bold Park population found five new seedlings, most likely germinating from seeds produced by the now mature plants that germinated after the 2000 fire (L. Hamilton, pers. comm.). The population at present requires monitoring and would benefit from augmentation (via cuttings) from material within Bold Park. However in the long term, if the population shows evidence of reduced viability then material could be introduced from Kings Park, the most genetically similar and geographically closest population.

Acknowledgements

All materials were collected under valid permits (DEC and BGPA). Thanks to R. Barrett, G. Messina, E. Bennett, B. Bowen, R. Dixon, F. Hort, A. Tinker, and L. Sage for help with locating field sites; K. Dixon and N. Swarts for samples from Nambung Flats; R. Standish and T. He for statistical advice; A. Shade and G. Messina for providing data on seed germination and propagation success; S. Easton for discussion on plant survival in the Kings Park bushland. This research was funded by an ARC linkage grant (LP0348958).

References

- Bennett, EM (1995) *Conospermum*. In 'Flora of Australia'. Vol. 16. (Ed P McCarthy) pp. 224–270. (CSIRO Australia: Melbourne.)
- BGPA (2000) Bold Park Environmental Management Plan 2000-2005. Botanic Gardens Park Authority, Bold Park, Western Australia.
- Bowen, BJ (1991) *Fire response within the family Proteaceae: a comparison of plants displaying the seeder and resprouter mode of recovery*. Unpublished PhD Thesis Department of Botany, University of Western Australia
- Broadhurst LM, Young AG, Thrall PH, Murray BG (2006) Sourcing seed for *Acacia acinacea*, a key revegetation species in south eastern Australia. *Conservation Genetics* **7**, 49-63.
- Bussell JD, Hood P, Alacs EA, Dixon KW, Hobbs RJ, Krauss SL (2006) Rapid genetic delineation of local provenance seed collection zones for effective rehabilitation of an urban bushland remnant. *Austral Ecology* **31**, 164-175.
- Butcher PA, Oteroa A, McDonald MW, Moran GF (2001) Nuclear RFLP variation in *Eucalyptus camaldulensis* Dehnh. from northern Australia. *Heredity* **88**, 402-412.
- Clarke KR, Gorley RN (2001) 'Primer v5: User Manual/Tutorial.' (Primer-E Ltd: Plymouth)

- Close DC, Messina G, Krauss SL, Rokich DP, Stritzke J, Dixon KW (2006) Conservation biology of the rare species *Conospermum undulatum* and *Macarthuria keigheryi* in an urban bushland remnant. *Australian Journal of Botany* **54**, 583 - 593.
- Cowling RM, Pressey RL, Rouget M, Lombard AT (2003) A conservation plan for a global biodiversity hotspot—the Cape Floristic Region, South Africa. *Biological Conservation* **112**, 191-216.
- Davis MB, Shaw RG (2001) Range shifts and adaptive responses to quaternary climate change. *Science* **292**, 673-679.
- Dell J, Banyard J (Eds) (2000) 'Bushland Forever.' (Department of Environmental Protection: Perth)
- Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variation inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.
- Goldingay RL, Carthew SM (1998) Breeding and mating systems in Australian Proteaceae. *Australian Journal of Botany* **46**, 421-437.
- Holsinger KE, Lewis PO (2003) 'Hickory: A package for analysis of population genetic data.' (University of Connecticut, Storrs)
<http://darwin.eeb.uconn.edu/hickory/hickory.html>
- Holsinger KE, Wallace LE (2004) Bayesian approaches for the analysis of population genetic structure: an example from *Platanthera leucophaea* (Orchidaceae). *Molecular Ecology* **13**, 887-894.
- Holsinger KE, Lewis PO, Dey DK (2002) A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology* **11**, 1157-1164.
- Hopper SD, Gioia P (2004) The Southwest Australian floristic region: Evolution and conservation of a global hot spot of biodiversity. *Annual Review of Ecology, Evolution and Systematics* **35**, 623-650.
- Houston, TF (1989) *Leioproctus* bees associated with Western Australian smoke bushes (*Conospermum* spp.) and their adaptations for foraging and concealment (Hymenoptera: Colletidae: Paracolletes). *Records of the Western Australian Museum* **14**, 275-292.

- Huff DR, Peakall R, Smouse PE (1993) RAPD variation within and among natural populations of outcrossing buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.). *Theoretical and Applied Genetics* **86**, 927-934.
- Jump AS, Peñuelas J (2006) Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. *Proceedings of the National Academy of Sciences, USA* **103**, 8096-8100.
- Krauss SL, He T (2006) Rapid genetic identification of local provenance seed collection zones for ecological restoration and biodiversity conservation. *Journal of Nature Conservation* **14**, 190-199.
- Krauss SL, Dixon B, Dixon KD (2002) Rapid genetic decline in a translocated population of the endangered plant *Grevillea scapigera*. *Conservation Biology* **16**, 1-11.
- Krauss SL, He T, Lamont BB, Miller BP, Enright NJ (2006) Late Quaternary climate change and spatial genetic structure in the shrub *Banksia hookeriana*. *Molecular Ecology* **15**, 1125-1137.
- Lande R, Shannon S (1996) The role of genetic variation in adaptation and population persistence in a changing environment. *Evolution* **50**, 434-437.
- Llorens TM, Ayre DJ, Whelan RJ (2004) Evidence for ancient genetic subdivision among recently fragmented populations of the endangered shrub *Grevillea caleyi* (Proteaceae). *Heredity* **92**, 519-526.
- Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology* **3**, 91-99.
- Miller MP (1997) Tools For Population Genetic Analysis (TFPGA) 1.3: A windows program for the analysis of allozyme and molecular population genetic data.
- Montalvo AM, Williams Rice SL, Buchmann SL, Cory C, Handel SN, Nabhan GP, Primack R, Robichaux RH (1997) Restoration biology: A population biology perspective *Restoration Ecology* **5**, 277-290.
- Morrison DA, McDonald M, Bankoff P, Quirico P (1994) Reproductive isolation mechanisms among four closely-related species of *Conospermum* (Proteaceae). *Botanical Journal of the Linnean Society* **116**, 13-31.

- Muluvi GM, Sprent JI, Soranzo N, Provan J, Odee D, Folkard G (1999) Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *Moringa oleifera* Lam. *Molecular Ecology* **8**, 463-470.
- Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature* **403**, 853-858.
- Newton AC, Allnut TR, Gillies ACM, Lowe AJ, Ennos RA (1999) Molecular phylogenetics, intraspecific variation and the conservation of tree species. *Trends in Ecology and Evolution* **14**, 140-145.
- Peakall R, Smouse PE (2001) 'GenAlEx V5: Genetic Analysis in Excel. Population genetic software for teaching and research.' (Australian National University: Canberra) <http://www.anu.edu.au/BoZo/GenAlEx/>
- Perry F, Trueman SJ (1999) Cutting propagation of Victorian smokebush, *Conospermum mitchellii* (Proteaceae). *South African Journal of Botany* **65**, 243-244.
- Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution* **49**, 1280-1283.
- Rousset F (1997) Genetic differentiation and estimation of gene flow from F-Statistics under isolation by distance. *Genetics* **145**, 1219-1228.
- Sainsbury RM (1991) 'A field guide to smokebushes and honeysuckles (*Conospermum* and *Lambertia*)'. (University of Western Australia Press, Perth).
- Sinclair EA, Hobbs RJ (accepted) Sample size effects on estimates of population genetic structure: implications for ecological restoration. *Restoration Ecology*.
- Sinclair EA, Bussell JD, Krauss SL, Hobbs R, Dixon KW (2006) Molecular markers detect multiple origins of *Agonis flexuosa* (Myrtaceae) plants used in urban bushland restoration. *Ecological Management and Restoration*. **7**, 234-235.
- Statsoft (1995) Statistica v4.1 for Macintosh. Statsoft, USA
- SPSS Incorporated, 2000. SPSS for Windows (v. 14.0). SPSS Incorporated, Chicago.
- van Andel J (1998) Intraspecific variability in the context of ecological restoration projects. *Perspectives in Plant Ecology, Evolution and Systematics* **1**, 221-237.
- Wright MG (1994) Unpredictable seed-set: a defence mechanism against seed-eating insects in *Protea* species (Proteaceae). *Oecologia* **99**, 397-400.

Figure captions

Fig. 1. Map of Western Australia showing the distribution of *Conospermum triplinervium* (modified from Florabase). The enlarged area shows sampling locations.

Fig. 2. A selection of leaf images from Kings Park plants showing the range in shape and size in *C. triplinervium*.

Fig. 3. X-ray images of *C. triplinervium* seeds showing a range in seed quality: Top row: viable, Middle row: undetermined (but most likely non-viable), Bottom row: non-viable.

Fig. 4. Two-dimensional MDS plots for *C. triplinervium* based on (a) six leaf measures (Stress = 0.09), and (b) 228 AFLP fragments (Stress = 0.22).

Table 1. Sample sites for *C. triplinervium*, giving latitude and longitude and climatic variables obtained from the Bureau of Meteorology.

Population	Sample Voucher	Bushland Forever Site number	Latitude	Longitude	Mean rainfall (mm)		Mean daily temperature	
			South	East	January	July	January	July
Nambung Flats	Ct158	-	30.518	-115.231	7.1	115.3	29.9	19.4
Julimar Forest	Ct92, Ct94(h*)	-	31.354	-115.216	4.5	75.4	30.4	15.4
Yanchep	Ct114	-	31.393	-115.692	8.8	128.8	29.0	19.1
Neerabup	Ct79	130	31.599	-115.709	8.8	128.8	29.0	19.1
Bold Park	Ct38	312	31.942	-115.772	12.1	153.3	31.4	18.5
Kings Park	Ct40	317	31.968	-115.836	18.1	165.7	30.6	18.2
Welshpool	Ct34	424	31.999	-115.964	8.8	161.9	31.6	17.8

* possible hybrid individual

Table 2. Mean and standard error (below in parentheses) for six leaf morphology measures in *C. triplinervium*.

Five leaves were collected from each plant.

Population	No. plants	No. leaves	Area (mm ²)	Elongation	Perimeter (mm)	Roundness	Major Axis Length (mm)	Minor Axis Width (mm)
Nambung Flats	26	130	791.04 (54.89)	9.29 (0.55)	224.86 (6.56)	0.20 (0.01)	100.21 (2.89)	11.75 (0.80)
Julimar Forest	20	100	691.69 (51.55)	14.41 (1.64)	267.32 (8.19)	0.13 (0.01)	114.58 (3.84)	9.36 (0.68)
Yanchep	13	65	1169.18 (66.33)	7.34 (0.64)	267.40 (11.17)	0.21 (0.01)	112.81 (5.42)	16.26 (1.01)
Neerabup	20	100	1132.64 (65.79)	9.43 (0.53)	273.24 (8.08)	0.19 (0.01)	123.22 (3.49)	13.81 (0.71)
Bold Park	4	20	1158.56 (64.56)	7.40 (0.61)	249.15 (3.84)	0.24 (0.02)	112.29 (2.35)	15.50 (0.86)
Kings Park	28	140	1512.77 (87.13)	7.18 (0.23)	284.06 (6.81)	0.23 (0.01)	128.01 (3.07)	18.48 (0.71)
Welshpool	32	160	1123.86 (37.70)	7.59 (0.12)	255.00 (4.86)	0.22 (0.00)	114.99 (2.16)	15.31 (0.28)

Table 3. Significant pairwise post-hocs tests using Scheffe's F procedure for post hoc comparisons between six leaf measurements for *C. triplinervium*. Alpha was significant at <0.00039 after Bonferroni correction for multiple pairwise tests.

Dependent Variable	Population 1	Population 2	Significance
Minor axis (width)	Julimar Forest	Welshpool	0.000002
	Julimar Forest	Kings Park	0.000000
	Nambung Flats	Kings Park	0.000000
	Julimar Forest	Yanchep	0.000013
Area	Julimar Forest	Kings Park	0.000000
	Nambung Flats	Kings Park	0.000000
Elongation	Julimar Forest	Welshpool	0.000000
	Julimar Forest	Kings Park	0.000000
	Julimar Forest	Yanchep	0.000010
	Julimar Forest	Nambung Flats	0.000257
Perimeter	Nambung Flats	Kings Park	0.000002
Roundness	Julimar Forest	Welshpool	0.000000
	Julimar Forest	Kings Park	0.000000
	Julimar Forest	Yanchep	0.000049
	Julimar Forest	Nambung	0.000030
	Julimar Forest	Neerabup	0.000329
Major axis (length)	Nambung Flats	Kings Park	0.000000

Table 4. Mean and standard deviation (below in parentheses) for five floral characters and seed viability counts in *Conospermum triplinervium*.

Population (coll. year)	Length of flower spike (mm)	Ave No. term. branches	Ave No. Flowers	Ave No. Seeds	Flower: Seed	% Seed set	Total Seeds	Viable seed	Undet. seed	Non-viable seed	% Viability	Insect bore holes*
Welshpool (2005)	277.53 (46.69)	11.85 (3.82)	11.20 (3.82)	1.78 (1.51)	7.53	16.46	1544	934	163	445	60	72
Bold Park (2005)	346.60 (74.34)	17.75 (6.56)	12.30 (4.12)	1.70 (1.49)	7.94	14.48	60	43	0	17	72	2
Kings Park (2005)	304.41 (62.33)	14.41 (6.12)	9.37 (5.07)	1.59 (1.54)	7.95	17.04	562	191	26	345	34	20
Kings Park (2006)	-	-	-	-	-	-	63	55	6	2	87	0

* All bore holes were in non-viable seeds, except one (undetermined).

Table 5. Number of polymorphic AFLP bands for *C. triplinervium* by sampling location and primer pair.

Population	N	Combined polymorphic bands		Polymorphic bands (%) by primer pair		
		N	%	<i>Pst</i> -AC	<i>Pst</i> -CT	<i>Pst</i> -CG
Nambung Flats	26	140	61.4	71.3	62.0	46.0
Julimar Forest	20	130	57.0	59.6	60.6	49.2
Yanchep	13	101	44.3	47.9	45.1	38.1
Neerabup	20	124	54.4	58.5	52.1	50.8
Bold Park	4	46	20.2	28.7	15.5	12.7
Kings Park	28	116	50.9	51.1	62.0	38.1
Welshpool	32	86	37.7	38.3	35.2	39.7
Mean per population	-	106	46.6	50.8	47.5	39.2
Overall	143	218	95.6	96.8	92.9	96.8

Table 6. P values for pairwise exact tests for population differentiation (Raymond and Rousset 1995) over all loci (P significant at 0.05; lower triangular matrix) and pairwise Theta-B (θ^B) values (upper triangular matrix) based on a Full model.

Population	1	2	3	4	5	6	7
Nambung Flats (1)	-	0.2186	0.2630	0.2316	0.2330	0.2804	0.6084
Julimar Forest (2)	0.0000	-	0.2187	0.2444	0.2763	0.3169	0.6149
Yanchep (3)	0.0000	0.0000	-	0.2712	0.2966	0.2963	0.6259
Neerabup (4)	0.0000	0.0000	0.0000	-	0.0595	0.1047	0.6366
Bold Park (5)	0.6378	0.1970	0.2188	1.0000	-	0.1492	0.6741
Kings Park (6)	0.0000	0.0000	0.0000	0.0207	1.0000	-	0.6524
Welshpool (7)	0.0000	0.0000	0.0000	0.0000	0.0007	0.0000	-

Table 7. DIC statistics for AFLP markers, by primer pair and overall data, for *C. triplinervium* under three alternative models. The smallest DIC model is highlighted in bold.

Marker	Model	Dbar	Dhat	pD	DIC
mCTT/ <i>Pst</i> -AC (n=94)	Full	1461.009	1149.4016	311.6074	1772.6163
	f = 0	1456.4467	1117.8681	338.5785	1795.0252
	$\theta^B = 0$	4711.0876	4624.0861	87.0014	4798.089
mCTT/ <i>Pst</i> -CT (n=71)	Full	1020.7322	789.1116	231.6205	1252.3527
	f = 0	1016.2343	767.1666	249.0677	1265.302
	$\theta^B = 0$	3068.8774	3009.3923	59.4851	3128.3624
mCTT/ <i>Pst</i> -CG (n=63)	Full	769.9746	606.7496	163.225	933.1997
	f = 0	768.3436	585.3113	183.0323	951.3759
	$\theta^B = 0$	2285.6188	2227.2357	58.3831	2344.0019
Overall (n=228)	Full	3257.2653	2526.7476	730.5176	3987.7829
	f = 0	3242.7309	2430.4954	812.2355	4054.9664
	$\theta^B = 0$	17280.2421	17063.7859	216.4562	17496.6983

20cm

(a)

(b)