Muelleria 18: 79–87 (2003)

Genetic comparison between Victorian and Tasmanian populations of *Prasophyllum correctum* D.L. Jones (Orchidaceae) suggests separate species

L. A. Orthia[1], R. C. Garrick[1] and E. A. James[1,2]

[1]Genetics Department, La Trobe University, VIC. 3086.
[2]National Herbarium of Victoria, Royal Botanic Gardens Melbourne, Private Bag 2000, VIC. 3141.

Abstract

Genetic variation within and between Tasmanian and Victorian populations of the Gaping Leek Orchid *Prasophyllum correctum* (Orchidaceae) was investigated using the Random Amplified Polymorphic DNA (RAPD) method. The degree of fixed genetic differences between the two populations was substantial, suggesting that each population constitutes a different species. The Tasmanian population contained very little genetic variation, indicating that asexual reproduction or self-fertilisation may be the predominant reproductive mode, but this population does not appear to be clonal. Individuals from the Victorian population exhibited high levels of genetic variation relative to those from the Tasmanian population. These findings suggest that the Victorian and Tasmanian *P. correctum* populations ought to be managed separately, and cross-pollination or translocation should be avoided, because of the lack of genetic similarity between them.
Keywords: *Prasophyllum*, RAPD, genetic variability, conservation

Introduction

The Gaping Leek Orchid, *Prasophyllum correctum* D.L. Jones, is a small terrestrial orchid from southeastern Australia. *Prasophyllum correctum* (Jones 1994) was believed to be endemic to Victoria until plants collected in 1995 from the Campbell Town golf course in Tasmania were identified as *P. correctum* (Jones 1998). The species is believed to have formerly been widespread throughout lowland Gippsland, but it is currently restricted to two small populations located near Munro and Lindenow South in protected rail reserves (Hoey & Lunt 1995) in *Themeda triandra* Forssk. grasslands or *Eucalyptus tereticornis* Sm. ssp. *mediana* grassy woodlands, which are recognised as endangered ecosystems in Victoria (Coates *et al.* 1999). In Tasmania, the species was known only from the Campbell Town golf course (Hoey & Lunt 1995; Jones 1998) until a single plant was found 7–8 km away in 1999 (Fig. 1). The presence of this outlier is considered to be reasonable confirmation that the species was more widespread before the Tasmanian midlands were opened up for agriculture (Threatened Species Unit 2000).

In Victoria, *Prasophyllum correctum* is listed as threatened under the *Flora and Fauna Guarantee Act* 1988 and has ROTAP code 2E (Briggs & Leigh 1996) with as few as 127 plants estimated to remain (Coates *et al.* 1999). The demise of the species has been attributed to habitat degradation through grazing and clearing for agriculture (Backhouse & Jeanes 1995; Hoey & Lunt 1995). Current threats to the continuing existence of Victorian *P. correctum* include rail reserve maintenance (e.g. rotary hoeing or slashing), grazing and trampling by roaming stock and feral animals, weed invasions, competition from native plant species, and herbicide use (Backhouse & Jeanes 1995; Hoey & Lunt 1995; Coates *et al.* 1999). In contrast, the Tasmanian population is estimated to contain at least 1000 individuals (Coates *et al.* 1999), and appears to be relatively stable in its unusual habitat of the Campbell Town golf course rough. But this population is still sufficiently threatened such that the species is listed as endangered under the Tasmanian *Threatened Species Protection Act* 1995, and under the federal *Environment Protection and Biodiversity Conservation Act* 1999. Although the site is not formally reserved, it is

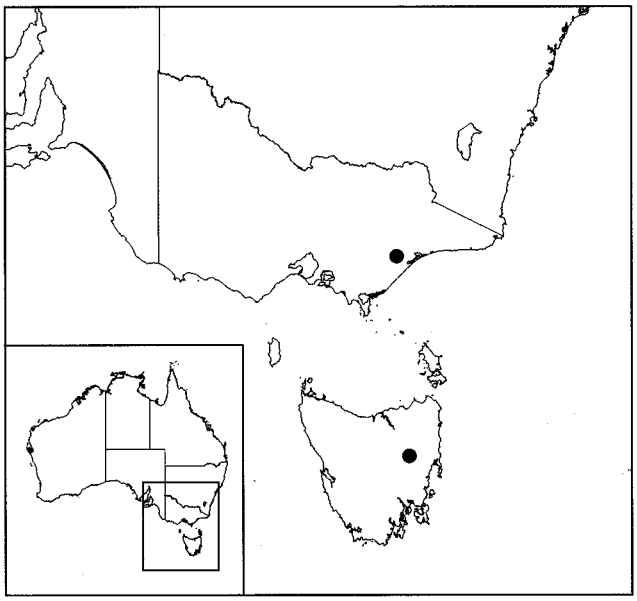

Figure 1. Known locations of *Prasophyllum correctum* and sites sampled for this study.

subject to a covenant agreement, which does provide some protection (Coates *et al.* 1999). Despite this, the population is still potentially threatened by activities that inadvertently contravene the covenant agreement such as use of fertilisers and recycled effluent (Coates *et al.* 1999).

The primary aim of the present study was to investigate the level of genetic diversity within and between populations of *P. correctum*. The *2000–2002 Recovery Plan* (Coates *et al.* 1999) proposed that such research should be undertaken to inform decisions pertaining to the collection of plant material for *ex situ* conservation programs. The *Recovery Plan* and the *Action Statement* for Victorian plants (Hoey & Lunt 1995) state that translocation of plants is an important management action. For this reason, gaining an understanding of the genetic variation present in both Victorian and Tasmanian populations would play a fundamental role in choosing plants from *ex situ* and *in situ* collections for translocation (Hoey & Lunt 1995; Coates *et al.* 1999). The *Recovery Plan* also noted that the Tasmanian *P. correctum* population contained more morphological variation than the smaller Victorian populations, and may therefore provide a useful reference point for gauging the level of diversity that may once have been present in Victoria.

In light of marked differences in the habitats presently occupied by Victorian and Tasmanian populations of *P. correctum*, and the large degree of geographic isolation between them, an additional aim of this study was to reassess their taxonomic relationship using a molecular approach. The Random Amplified Polymorphic DNA (RAPD) method has previously been used successfully to indicate the level of genetic diversity within populations, between populations and between taxa (e.g. Boehm *et al.* 1993; Sulaiman & Hasnain 1996; Qamaruz-Zaman *et al.* 1998; Tyson *et al.* 1998; Gillies *et al.* 1999; Coleman *et al.* 2000; van der Nest *et al.* 2000; Warburton *et al*. 2000). The RAPD method was chosen because it enables the rapid identification of polymorphic loci that are useful in population-genetic studies and for reflecting taxonomic distinctions, yet requires no prior information on the genetics of the organism. The latter attribute of RAPD is particularly advantageous for work on species for which few genetic data are available, including the present orchid species.

Materials and Methods

POPULATION SAMPLING

In Victoria, *P. correctum* plant material was collected from the largest known population, near Munro. Samples of leaf tissue were collected in August and October 2001 from 15 plants that are the subjects of a long-term monitoring program. Leaf tissue samples were collected from ten individuals at each of the three Campbell Town golf course subpopulations in September 2001. At all sites, leaf material was initially wrapped in moist paper towel and kept in sealed plastic bags on wet ice. Tissue samples were subsequently stored at –86ºC until DNA was isolated.

DNA ISOLATION

A section of tissue 2–3 cm long, weighing between 50 and 180 mg was removed from each leaf sample for DNA extraction. All selected leaf sections appeared to be disease free, and were taken from the leaf tip to reduce the risk of sampling regions contaminated by pathogenic or mycorrhizal organisms. Tissue was ground to a fine powder in liquid nitrogen using a ceramic mortar and pestle. Ground material was collected in a pre-chilled 1.5 ml microcentrifuge tube. All samples were assigned a unique alpha-numeric label where the letter refers to the collection locality ('V': Victoria or 'T': Tasmania), and the number refers to an individual plant.

Genomic DNA was extracted using a QIAGEN DNeasy® Plant Mini Kit following the manufacturer's instructions, except 3 µl (rather than 4 µl) of RNase A stock solution was added to each tissue sample in step 2; and DNA was eluted in 75 µl (rather than 100 µl) of elution buffer in steps 12 and 13.

PCR AND ELECTROPHORESIS

DNA was amplified in 20 µl reactions containing 10 µl QIAGEN HotStarTaq™ Master Mix, 9 µl dH_2O, approximately 0.25 µM primer and 1 µl (5-20 ng) of template DNA. Polymerase Chain Reactions (PCR) were performed in an Eppendorf Mastercycler® gradient thermal cycler using the following profile: 95ºC for 15 mins, 35ºC for 2 mins; 72ºC for 90 s (1 cycle), 94ºC for 30 s, 38ºC for 30 s, 72ºC for 30 s (35 cycles) with a final extension step of 72ºC for 4 mins 30 s (1 cycle).

A few *P. correctum* individuals were screened for anonymous polymorphic loci using 22 RAPD primers (Operon Technologies, OPA, OPB and OPF series PCR primers). Of the 16 RAPD primers that yielded amplification products, six were randomly selected for population-genetic assessment of *P. correctum*: OPA–03, OPA–04, OPA–13, OPF–04, OPF–09 and OPF–14. Samples of DNA from all individuals were amplified simultaneously for each primer, and a negative control was included in each PCR to facilitate identification of contamination. A small number of samples were rerun either to check that band patterns were reproducible, to clarify poorly amplifying samples, or to compare relative sizes of bands from different individuals. Amplification products were separated via electrophoresis on 1.5% agarose gels stained with ethidium bromide in 1x TBE running buffer at 80 V for 30–120 min (depending on the size of the gel).

SCORING BAND PATTERNS

Gels were viewed with ultraviolet light and photographed either with a Polaroid camera or a Kodak EDAS 120 digital camera. Kodak 1D Version 3.5 imaging software was used to estimate band sizes in digital images and all gels were also scored by eye. Bands were scored as absent (0) or present (1). Duplicate PCR runs were conducted for most sample/primer combinations, and any samples that produced faint or ambiguous bands were rerun. Very faint or ambiguous bands were omitted from the dataset.

ANALYSIS

The dataset was analysed using Genstat for Windows (Version 4.2). A pair-wise similarity matrix between individuals was constructed using Jaccard's coefficient in order to

analyse the relationship between the two populations. The matrix was then used as the basis for ordination by principal coordinate analysis (Gower 1966). The Shannon Diversity Index was calculated for each population and the species as a whole using POPGENE V# (Yeh *et al*. 1997). The index allows a comparison of the degree of variation within each population and is appropriate where Hardy-Weinberg equilibrium cannot be assumed. Partitioning of variation within and among populations was calculated from the index (King & Schaal 1989).

Results

In total, 72 bands were scored for the six primers. Primer OPA–04 produced 4 bands; all others produced between 11 and 15 bands ranging in size from approximately 300 to 2000 bp. Only six bands were common to both Victorian and Tasmanian populations, and five of those were from one primer, OPF–04. The sixth common band (OPF–14) was present in 11 Victorian individuals but only in one Tasmanian plant (T23) (Fig. 2 & 3).

Overall, the Victorian specimens showed a far higher degree of variation than specimens from Tasmania (Fig. 2 & 3). Within the Tasmanian population, 32 bands were present. Fourteen (44%) of those were polymorphic, but 11 of the polymorphic bands were present only in one or two individuals, and often in the same few individuals, leaving a large proportion of the population appearing to be genetically identical. Thirteen individuals showed identical band patterns for all six primers, and another four identical individuals all differed from those 13 by just one band. A further seven individuals were identical to each other and differed from the 13 by two bands and from

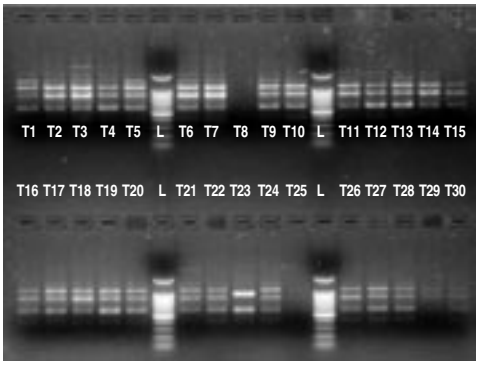

Figure 2. Band patterns of Tasmanian plants for OPF–14. Those that did not amplify well or at all were successfully amplified later. It can be seen from this that band patterns were very uniform, with only one or two polymorphisms (eg T23). The brightest band in the T23 pattern was the only band Tasmanian plants shared with Victorian plants, along with those produced by OPF–04 (not shown).

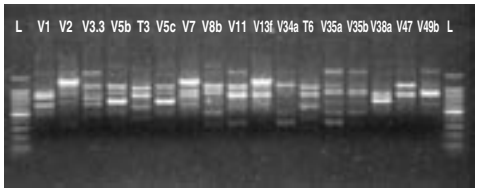

Figure 3. Victorian specimens amplified with OPF–14 and interspersed with Tasmanian specimens to compare band sizes. Note the amount of variation displayed by the Victorian plants compared with the Tasmanian plants in Figure 2.

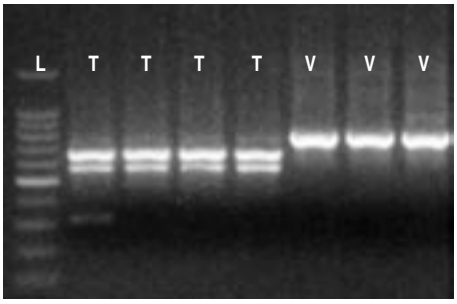

Figure 4. A subset of samples amplified with primer OPA–04. All Victorian samples produced the same pattern as those shown here; all Tasmanian samples showed the top two bright bands shown here, and about half also showed the lower faint band.

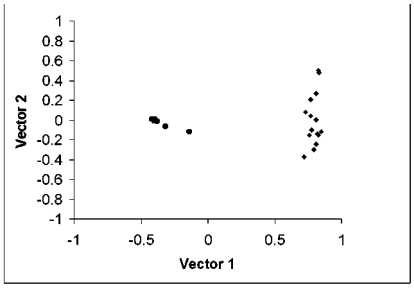

Figure 5. Ordination plot for *P. correctum* showing a strong separation of Tasmanian (●) and Victorian (◆) populations.

the four by one band. Of the remaining six plants, four only differed from the majority by one polymorphism. The other two individuals, T23 and T30, shared four polymorphic states, plus T23 showed six RAPD characters not shared by any other Tasmanian individuals. In other words, most of the variation present was represented by one individual, T23.

In contrast to this striking genetic uniformity among Tasmanian *P. correctum* individuals, the Victorian plants showed great diversity. Of the 46 bands present among Victorians, 38 (83%) were polymorphic. No two individuals showed the same band pattern. This was consistently the case for all primers but one. The exception, OPA–04, produced identical patterns for all Victorian plants (Fig. 4). The minimum difference between two individuals was one band, and the maximum difference was 21 bands.

The analysis of these data using ordination techniques (Fig. 5) showed a very distinct separation between Tasmanian and Victorian plants. Vector 1 contained 64% of the variation present. The Shannon Diversity Index for the Tasmanian population was H_O = 0.076 compared to H_O = 0.236 for the Victorian population indicating a much greater relative genetic diversity in the Victorian population. Partitioning of variation indicates that 65.3% of variation is attributable to among-population variation.

Discussion

GENETIC VARIATION AND TAXONOMY

The distinct genetic separation of the two populations illustrated in the ordination suggests that the Tasmanian and Victorian plants not only share very little genetic variation, but in fact belong to different taxa. The fact that the two populations shared so few RAPD bands suggests that if Tasmanian and Victorian plants share a common

ancestor, they have been genetically separated for a long time, and have had the opportunity to acquire population-wide mutations at many sites in the genome. Alternatively, their origins might involve a complex taxonomic history. The amount of time since the two species can be traced back to a common ancestor cannot be calculated from RAPD data. This may be an area of further study if the taxonomic status of the two populations remains in doubt (see Jones, this issue).

RAPD bands that did appear to be shared by both populations (Fig. 2 & 3) were similar in size but their homology cannot be ascertained absolutely without DNA hybridisation tests (see Rossetto *et al.* 1996). No Victorian plant has a band pattern identical to any of the Tasmanian plants. It seems tenuous at best to maintain that the two populations are one species given this paucity of evidence. Had our starting point been the assumption that the two populations were different species, we would not have considered the six shared bands to be evidence of shared taxonomic status.

In orchids, the RAPD technique consistently detects genetic variation at the specific and interspecific level even. A study of Italian taxa of the *Ophrys bertolonii* aggregrate, separated mainly on subtle differences in morphological characters, found high levels of genetic variability within populations (Grünanger *et al.* 1998). Distinct species were successfully separated, at a level of 80% similarity using Jaccard's coefficient, but only one of the aggregate species could be distinguished prompting the authors to recognise only a single taxon from the remaining taxa in the aggregrate. In contrast, the two populations of *P. correctum* showed a similarity of 40% based on the simple matching coefficient. The evolution of *Spiranthes hongkongensis* by natural hybridisation and polyploidisation of *S. sinensis* and another species was also supported by RAPD analysis (Chan & Sun 1997).

The low amount of genetic variation present in the Tasmanian population begs discussion. While the population does not appear to be strictly clonal, it seems that either asexual reproduction or inbreeding is common, the population started its existence with only a small amount of genetic variation, or the species has existed in a very stable habitat over a long period of time (James 2000). It has been noted by Bates (1994) that at least one *Prasophyllum* species, *P. goldsackii*, has been observed to produce seed without fertilisation (apomixis) although this has not been verified. Clements (1995) showed that apomixis occurred in the Australian terrestrial orchid *Corunastylis apostasiodes*, a related genus in the subtribe Prasophyllinae. Dixon (1991) observed asexual reproduction by distal daughter tuber formation for some *Prasophyllum* species. It is also possible that the Tasmanian plants are part of a founder colony, begun by very few plants with a reduction in genetic diversity resulting from inbreeding and the accumulation of few mutations since foundation. It is theoretically possible that the population was founded many years ago by a single Victorian plant of *P. correctum*. The Victorian population is variable enough to allow for the possibility that the standard Tasmanian genetic pattern began as a single Victorian plant that has since diverged. However, evidence against this includes the highly conserved regions amplified by OPA–04 that differ strongly between the populations.

Primer OPA–04 (Fig. 4) provides some of the strongest evidence for separating the two populations taxonomically. It showed the least amount of within-population variation, with no differences observed between Victorian individuals. Tasmanian plants showed variation at one band, making this the only primer to show more variation among Tasmanian plants than among Victorian plants. The low amount of within-population variation suggests that sites in the genome that were amplified using this primer were more highly conserved than many of the sites amplified using other primers. If highly conserved sites are not shared, then the two populations are more likely to have evolved as separate species in the distant past, rather than to have diverged more recently.

The high degree of genetic variation present in the Victorian population is clearly the opposite of the expectation expressed in the *Recovery Plan* (Coates *et al.* 1999). Loss of

genetic variation may not be critical yet for the Victorian plants, despite the small size of the populations. Typically, *P. correctum* plants are long-lived, with their tubers being replaced after flowering each year and they can also lie dormant for up to 5 years (Coates *et al.* 1999). There may be more variation present than the genetic analysis revealed because a different combination of plants emerges each year. The genetic diversity revealed in this study may be a reflection of the diversity present when the species was more abundant. But, any detrimental effects of inbreeding may not yet be readily identified.

IMPLICATIONS FOR CONSERVATION MANAGEMENT OF THE SPECIES

The Victorian and Tasmanian populations of *Prasophyllum correctum* must certainly be managed separately and should not be cross-pollinated in *ex situ* collections or translocated. Even if they are not confirmed as different species, they should be considered to be evolutionarily significant units, worthy of separate conservation measures, because of the paucity of overlap in genetic variation.

Jones (1991) noted that *Prasophyllum* species in general routinely present a multitude of taxonomic problems including species complexes. The present-day disjunct distribution of populations that are currently recognised as *P. correctum* may be the result of a vicariance event such as the disappearance of the mainland-Tasmanian land bridge that crossed Bass Straight 13,000 years ago (White 1994) or establishment of populations by for example, long-range seed dispersal. Alternatively, it is possible that although individuals from Victorian and Tasmanian populations are presently morphologically very similar, they actually originated from different evolutionary lineages with their similarity resulting from convergent evolution.

There are risks associated with crossing individuals that are genetically too dissimilar. Long isolated populations may have developed adaptations, specific to particular regions or habitats, that break down by reassortment and recombination if populations hybridise. This can result in outbreeding depression whereby offspring have reduced fitness for example, low vigour and reduced fertility (Barrett & Kohn 1991). Consequently, conservation strategies that involve transplanting individuals from one population to another, encourage gene flow between geographically isolated populations, or store *ex situ* propagules from different geographic locations together, should be approached with caution. Such habitat-specific adaptations have been documented in *Prasophyllum* species (see Jones 1991). In addition, species integrity may be lost by hybrid swamping if different species inadvertently interbreed with closely related taxa (Riesberg 1991). This is particularly pertinent for orchids, which usually evolve ecological or mechanical barriers to hybridisation rather than physiological barriers, making them amenable to interspecific and even intergeneric hybridisation (IUCN 1996). For *Prasophyllum*, however, there is little evidence for species-specific pollen vectors and *P. correctum* has easily accessible pollen with removal possible by a number of vectors (Rouse, pers. comm. 2003). Although ecological barriers to hybridisation such as species-specific pollinator-plant relationships may be highly effective under natural conditions, it is possible that pre-zygotic mechanisms would break down under altered environmental conditions. One such situation might include human-mediated plant introductions for example, translocation of plants between geographically isolated areas such as Victoria and Tasmania. The generally degraded state of the Victorian *P. correctum* habitat could also contribute to reduced integrity of traditional pollination systems.

The Victorian population at Munro appears to have retained a significant amount of the genetic variation presumed to have been present when the species' population sizes were larger. However, the small number of individuals makes the Victorian population likely to be more at risk from habitat destruction and stochastic environmental events than from inbreeding depression or loss of adaptive ability (c.f. the Tasmanian population). It is therefore a priority to establish *ex situ* collections of the species and a translocation

and/or reinforcement program to increase the number of plants growing in Victoria. Coates (2001) has proposed measures to maximise the survival and fecundity of *in situ* plants, such as imposing a regular fire cycle on the sites based on data showing that fire intervals of 2–3 years are beneficial. These measures should be both heeded and implemented, in order to retain as much variation as possible in the wild.

Despite evidence for other species whose limited variation does not appear to be detrimental (James 2000), the minimal genetic variation in the Tasmanian population is cause for concern. Low variation is likely to reduce the ability of the population to respond to changing environmental conditions and selective pressures, thus increasing its risk of extinction. The *Recovery Plan* noted that the Tasmanian plants showed considerable floral variation not evident in Victoria (Coates *et al.* 1999), but this was not reflected in the present genetic data. Further genetic surveys of the Tasmanian plants, combined with tagging and monitoring of individuals, would be useful for maximising the diversity within material collected for *ex situ* propagation. In addition, elucidation of the breeding system for the Tasmanian plants and data on seed production will assist in the development of guidelines for collection of propagules.

In summary, the evidence is strong that the Tasmanian and Victorian *P. correctum* plants should be classified as separate species. Further morphological study (Jones, this issue) has confirmed that reclassification is appropriate. Sampling for *ex situ* material can be based on the population genetic structure found in this study. Information on the reproductive biology of both Victorian and Tasmanian plants can be used to minimise the risk of losing genetic diversity in any seedlings produced from a breeding program based on *ex situ* plants.

Acknowledgments

Thanks to Roger Riordan and the Royal Botanic Gardens Melbourne, specifically the Plant Science and Biodiversity Division, for the opportunity for two of us (LAO and RCG) to pursue this project as part of the Jim Willis Studentship program. Thanks to Hans Wapstra, Josephine MacHunter and Fiona Coates for help collecting plant material and insights into the species. Thanks also to Cassandra McLean and members of her lab at Burnley for access to their digital imaging system.

References

Backhouse, G. and Jeanes, J. (1995). *The Orchids of Victoria*. Melbourne: Miegunyah Press.

Barrett, S.C.H. and Kohn, J.R. (1991). Genetic and evolutionary consequences of small population sizes in plants: Implications for conservation. *Genetics and Conservation of Rare Plants*. Oxford University Press: New York.

Bates, R.J. (1994). *Prasophyllum*. In Walsh, N. G. and Entwisle, T. *Flora of Victoria, Volume 2: Ferns and Allied Plants, Conifers and Monocotyledons*. Melbourne: Inkata Press.

Boehm, M., Loew, R., Haag-Kerwer, A., Luettge, U. and Rausch, T. (1993). Evaluation of comparative DNA amplification fingerprinting for rapid species identification within the genus *Clusia*. *Botanica Acta* **106**, 448–453.

Briggs, J.D. and Leigh, J.H. (1996). *Rare or Threatened Australian Plants*. (CSIRO Publishing, Collingwood).

Chan, M.C. and Sun, M. (1997). RAPD and chloroplast DNA evidence for allopolyploid speciation of *Spiranthes hongkongensis* (Orchidaceae). *American Journal of Botany* **84**, 115.

Clements, M.A. (1995). Reproductive biology in relation to phylogeny of the Orchidaceae especially the tribe Diurideae. Ph.D. Thesis. Australian National University, Canberra.

Coates, F. (2001). *Patterns of appearance and response to management of Prasophyllum correctum D.L.Jones at Munro, Victoria.* Unpublished report to the Department of Natural Resources and Environment, East Melbourne.

Coates, F., Lunt, I. and Wapstra, H. (1999). *Draft Recovery Plan 2000–2002 – Prasophyllum correctum (Gaping Leek Orchid).* School of Botany, La Trobe University, Victoria.

Coleman, M., Hollingsworth, M.L. and Hollingsworth, P.M. (2000). Application of RAPDs to the critical taxonomy of the English endemic elm, *Ulmus plotii* Druce. *Botanical Journal of the Linnean Society* **133**, 241–262.

Dixon, K. (1991). Seeder/clonal concepts in Western Australian orchids. In: *Population Ecology of Terrestrial Orchids*, ed. T. C. E. Wells and J. H. Willems. Pp111–124. The Hague: SPB Academic Publishing.

Gillies, A.C.M., Navarro, C., Lowe, A.J., Newton, A.C., Hernandez, M., Wilson, J. and Cornelius, J.P. (1999). Genetic diversity in Mesoamerican populations of mahogany (*Swietenia macrophylla*), assessed using RAPDs. *Heredity* **83**, 722–732.

Gower, J.C. (1966). Some distance properties of latent root and vector methods used un multivariate analysis. *Biometrika* **53**, 325–338.

Grünanger, P., Caporali, E., Marziana, G. Menguzzato, E. and Servettaz, O. (1998). Molecular (RAPD) analysis on Italian taxa of the *Ophrys bertolonii* aggregate (Orchidaceae). *Plant Systematics and Evolution* **212**, 177–184.

Hoey, J. and Lunt, I. (1995). Gaping Leek-orchid *Prasophyllum correctum.* In: Flora and Fauna Guarantee action statements vol. 1, no. 57. East Melbourne, Vic.: Dept of Conservation & Natural Resources. Flora & Fauna Branch.

IUCN / SSC Orchid Specialist Group. (1996). *Orchids – Status Survey and Conservation Action Plan.* Gland, Switzerland and Cambridge, UK: IUCN.

James, S.H. (2000). Genetic systems in the south-west flora: implications for conservation strategies for Australian plant species. *Australian Journal of Botany* **48**, 341–347.

Jones, D.L. (1991). New Taxa of Australian Orchidaceae. *Australian Orchid Research* **2**, 1–207.

Jones, D.L. (1994). A new name for the Australian orchid *Prasophyllum chasmogamum* R.Bates & D.L.Jones (Orchidaceae). *Novon* **4**, 106–108.

Jones, D.L. (1998). Contributions to Tasmanian Orchidology 1–9. *Australian Orchid Research* **3**, 1–224.

King, L.M. and Schaal, B.A. (1989). Ribosomal-DNA variation and distribution in *Rudbeckia missouriensis. Evolution* **43**, 1117-1119.

Qamaruz-Zaman, F., Fay, M.F., Parker, J.S. and Chase, M.W. (1998). Molecular techniques employed in the assessment of genetic diversity: A review focusing on orchid conservation. *Lindleyana* **13**, 259–283.

Riesberg, L.H. (1991). Hybridization in rare plants: Insights from case studies in *Cercocarpus* and *Helianthus*. *Genetics and Conservation of Rare Plants*. Oxford University Press: New York.

Rossetto, M., Lucarotti, F., Hopper, S.D. and Dixon, K.W. (1996). DNA fingerprinting of *Eucalyptus graniticola*: a critically endangered relict species or a rare hybrid? *Heredity* **79**, 310–318.

Sulaiman, I.M. and Hasnain, S.E. (1996). Random amplified polymorphic DNA (RAPD) markers reveal genetic homogeneity in the endangered Himalayan species *Meconopsis paniculata* and *M. simplicifolia*. *Theoretical and Applied Genetics* **93**, 91–96.

Threatened Species Unit. (2000). *Listing statement Gaping leek orchid Prasophyllum correctum*. Department of Primary Industries, Water and Environment, Tasmania.

Tyson, M., Vaillancourt, R.E. and Reid, T.B. (1998). Determination of clone size and age in a mallee eucalypt using RAPDs. *Australian Journal of Botany* **46**, 161–172.

van der Nest, M.A., Steenkamp, E.T., Wingfield, B.D. and Wingfield, M.J. (2000). Development of single sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). *Mathematical Biosciences* **168**, 433–436.

Warburton, C.L., James, E.A., Fripp, Y.J., Trueman, S.J., and Wallace, H.M. (2000). Clonality and sexual reproductive failure in remnant populations of *Santalum lanceolatum* (Santalaceae). *Biological Conservation* **96**, 45-54.

White, M.E. (1994). *After the Greening: The browning of Australia*. Kangaroo Press: Sydney.

Yeh, F.C. and Boyle, T.J.B. (1997). Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**, 157.