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Genetic comparison of populations of the rare Gorae Leek Orchid, *Prasophyllum diversiflorum* Nicholls (Orchidaceae)

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Abstract

The rare Gorae Leek Orchid, *Prasophyllum diversiflorum*, is listed as a threatened species under Victorian legislation. Information on its patterns of genetic variation is urgently required to develop effective conservation strategies for the species. The two remaining populations of *P. diversiflorum* were compared using the Random Amplified Polymorphic DNA (RAPD) method. Despite small population sizes, the level of genetic diversity detected was encouraging for the long-term survival of the species. Both populations showed similar levels of genetic variation. There was little population differentiation with 88% of genetic variation residing within populations.

Key words: conservation; population genetics; *Prasophyllum diversiflorum*; RAPD.

Introduction

The Gorae Leek Orchid, *Prasophyllum diversiflorum* Nicholls, a terrestrial orchid endemic to south-west Victoria, is currently listed as threatened under the Victorian *Flora and Fauna Guarantee Act* 1988 and "endangered 2E" nationally (Briggs & Leigh 1996). The species exhibits considerable variation in floral morphology (Nicholls 1942), and grows along natural watercourses, around swamp margins and on seasonally inundated alluvial flats in open forest or grassy woodland vegetation (Backhouse & Jeanes 1995, Bishop 1996, Rouse 2002). Colonies were once known to have flourished over an area of several hundred acres at Gorae West (Nicholls 1942). But just seven years after the initial discovery of *P. diversiflorum* in 1941, the species was presumed to have become extinct owing to the conversion of its natural habitat to agricultural land. The species was rediscovered 35 years later in the Condah-Hotspur district, 40 km from the type locality (Backhouse & Jeanes 1995, Bishop 1996). Today, *P. diversiflorum* is known from only two small populations, near Dunkeld and Hotspur, which consist of approximately 800 plants and 250 plants, respectively (Rouse 2002).

The taxonomy of the group to which *P. diversiflorum* belongs is complex and there have been differences of opinion as to which populations constitute the species. For example, the *2000-2004 Recovery Plan* (Ingeme & Govanstone 1999) lists six populations, but at this stage, only two remaining *P. diversiflorum* populations can be confirmed. Neither is on reserved land (Rouse 2002). Current threats to the species' survival include grazing by livestock, competitive exclusion by exotic plants, herbicide spraying, and road construction works (Backhouse & Jeanes 1995).

Genetic data is now widely used to guide conservation and recovery programs of endangered plant species (Godt & Hamrick 1999). Yet despite the high percentage of orchid species that are endangered, genetic diversity of orchid taxa has received scant attention (Wong & Sun 1999). Information about a species' genetic diversity can provide useful baseline data for conservation (Geburek 1997) and is often a valuable component of comprehensive conservation management strategies (Hamrick 1983, Falk & Holsinger 1991). Certain aspects of conservation biology, including the loss of genetic diversity in

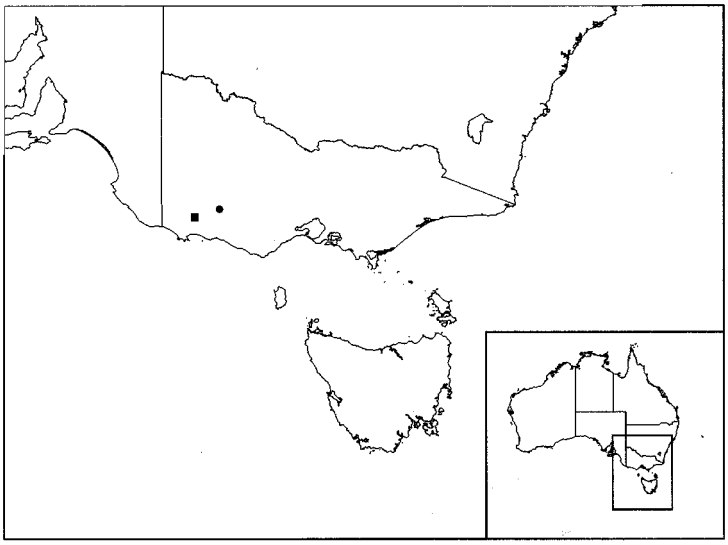

Figure 1. Map of *Prasophyllum diversiflorum* collection localities used in this study (● Dunkeld, ■ Condah-Hotspur).

conservation programs and the restoration of threatened populations, necessarily require detailed population-genetic studies (Hamrick & Godt 1995).

There is virtually no information on the biology and ecology of the Gorae Leek orchid (Ingeme & Govanstone 1999). An objective of the *2000-2004 Draft Recovery Plan* was to investigate factors affecting plant recruitment and population viability including the genetic health and viability of the remnant populations. Information regarding the amount and the partitioning of genetic variation within and between *P. diversiflorum* populations may yield insights into the degree of genetic similarity between the two populations and provide guidance for the species' conservation management.

The apparent ecological specialisation of *P. diversiflorum*, combined with past and present anthropogenic threats, and very small population sizes, make the survival of this species of immediate concern. It is a prime candidate for *ex situ* conservation measures (Backhouse & Jeanes 1995), but prior to the present research, no information has been available to assist decision-making about whether for example, plants from different populations can be safely cross-fertilised with one another.

The aim of this study was to investigate the level of genetic diversity present within and between the two known populations of *P. diversiflorum* and to compare their genetic similarity. The Random Amplified Polymorphic DNA (RAPD) method (Williams *et al.* 1990) was used as it has been successfully employed in the past to indicate levels of genetic diversity within populations, between populations and between taxa in both orchids and other plant species (e.g. Boehm *et al.* 1993, Sulaiman & Hasnain 1996, Qamaruz-Zaman *et al.* 1998, Wong & Sun 1999). The information will assist decision-making regarding the source of germplasm used to establish an *ex situ* collection and breeding program, as well as manipulation of reproduction at field sites.

Materials and Methods

COLLECTION OF PLANT TISSUE

In January 2002, three flowers were collected from inflorescences of 15 individuals at each of the two *P. diversiflorum* populations located near Dunkeld and along the Condah-Hotspur Road, Hotspur, in south-western Victoria (Fig. 1). Each sample was assigned a

unique alpha-numeric label where the letter refers to the collection locality (D: Dunkeld, H: Condah-Hotspur), and the number refers to a particular plant.

ISOLATION AND AMPLIFICATION OF GENOMIC DNA

Plant material was ground in liquid nitrogen using a mortar and pestle. Two flowers per individual were ground up together in order to increase DNA yield following inspection of ovaries to ensure samples had not been fertilised. Genomic DNA was isolated using a QIAGEN DNeasy® Plant Mini Kit following the protocol recommended by the manufacturer with two modifications: 3 µl (not 4 µl) of RNase stock solution was added to each tissue sample (step 2), and 75µl (not 100 µl) of Buffer AE was used to elute DNA (step12 and step 13). Amplification of DNA via Polymerase Chain Reaction (PCR) was performed in 20 µl reactions containing 10 µl Qiagen HotStar Taq® Master Mix, 8.2 µl Millipore dH_2O, 0.8 µl primer and 1 µl template DNA (10-30 ng). All amplifications were performed in an Eppendorf Mastercycle® gradient cycler with the following profile: 15 min at 95ºC, 2 min at 35 ºC, 90 s at 72 ºC (1 cycle), 30 s at 94 ºC, 30 s at 38ºC, 30 s at 72 ºC (35 cycles) with a final extension step of 4 min 30 s at 72 ºC (1 cycle). A negative control was included in each PCR run to facilitate identification of contamination.

RAPD ANALYSES

Thirty-eight RAPD primers (Operon Technologies) were assessed. Five primers (OPA-02, OPF-03, OPF-05, OPF-09, OPF-13) were selected from 16 primers that consistently yielded well-resolved polymorphic PCR amplification products. PCR products were separated via electrophoresis on 1.5% agarose gels using a 1µ TBE electrode buffer (90 mM Tris-borate, 2 mM EDTA). Gels were run at 80 V for 30-120 min depending on gel size. PCR products were stained with ethidium bromide then visualised under ultraviolet light at 302 nm. Duplicate PCR runs were conducted to confirm reproducibility of RAPD bands. Matching samples from two independent PCR runs were run together side by side, for all individuals. Gels were photographed with a Polaroid GelCam or Kodak digital camera and DNA fragment sizes were estimated using Kodak Digital Analysis System 120 gel analysis software. All fragment size estimates and RAPD profiles were checked by eye, and any poorly resolved or consistently ambiguous bands were omitted from the data set.

STATISTICAL ANALYSES

The presence or absence of DNA fragments was recorded for each sample in a binary matrix. A similarity matrix using the Simple Matching coefficient was calculated and used as the basis for ordination by principal co-ordinates (Gower 1966) using the program Genstat 5 for Windows (Lawes Agricultural Trust Rothamstead). Shannon's Diversity Index was calculated for each population and for the species using POPGENE software (Yeh & Boyle 1997) and the amount of variation partitioned within and between the populations was derived from them (King & Schaal 1989).

Results

Forty-nine bands were scored for the five RAPD primers used in this study. The number of bands per primer varied from five to 15 and ranged in size from approximately 290 to 2000 base pairs. All but two bands were common to both *P. diversiflorum* populations, so the vast majority of differences between populations resided in the frequency of RAPD fragments rather than the presence of population-specific fragments.

Overall, there was a high degree of genetic variation within the Dunkeld and Condah-Hotspur populations. RAPD profiles from OPF-13 for all individuals are shown in Figure 2. While there is variation within both *P. diversiflorum* populations, it is clear that bands are also shared between populations. The reduced similarity matrix (Table 1) shows the

Table 1. Reduced similarity matrix (%) based on RAPD fragments for the two populations of *P. diversiflorum*.

Population	Dunkeld	Condah-Hotspur
Dunkeld	74.6	
Condah-Hotspur	70.4	74.7

Table 2. Comparison of shared bands (above diagonal) and % similarity (below diagonal) based on RAPD fragments for primer OPF-09 for the two populations of *P. diversiflorum* and the Victorian population of *P. correctum* at Munro.

Population	Dunkeld	Condah-Hotspur	*P. correctum*
Dunkeld	-	8	5
Condah-Hotspur	71.6	-	5
P. correctum	49.9	55.5	-

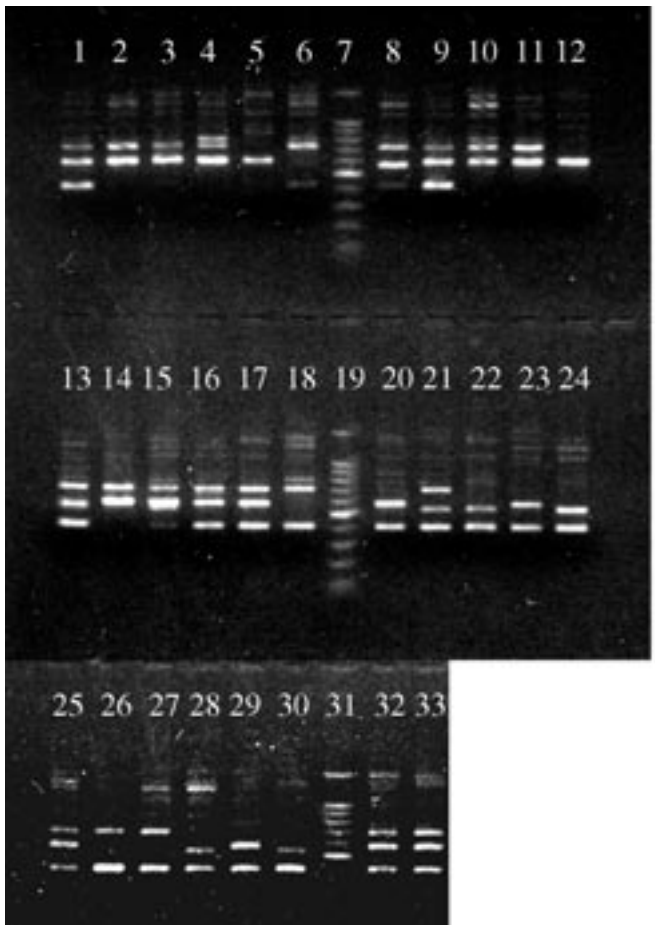

Figure 2. All samples amplified with primer OPF13. Dunkeld: lanes 1-6, 8-16. Condah-Hotspur: 17-18, 20-30, 32-33. Promega 100bp ladder: 7, 19, 31.

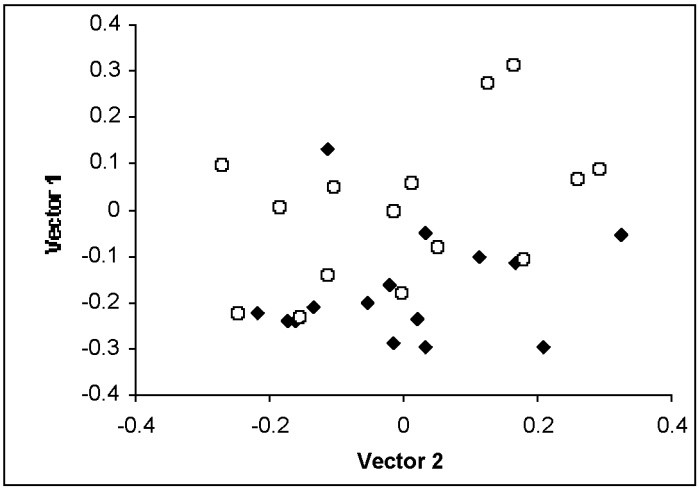

Figure 3. Ordination of RAPD data for *Prasophyllum diversiflorum*. (◆ Dunkeld, O Condah-Hotspur)

age similarity drops only marginally from 75% to 70% when comparing individuals from different populations. In contrast, when RAPD data for OPF-09 are compared, similarity between *P. diversiflorum* and the Victorian population of *P. correctum* drops from 71.6% between the two *P. diversiflorum* populations to 55.2% and 49.9% for Dunkeld and Condah-Hotspur, respectively, (Table 2).

The Shannon Diversity Index, H_O, was very similar for both Dunkeld and Condah-Hotspur populations, 0.357 and 0.364, respectively. Nei's genetic identity was 0.9267 with 88.0% of the total variation attributable to within-populations variation. The gene flow estimate was $N_m = 4.2$.

Ordination of these data shows that while there is some grouping of plants by population, the *P. diversiflorum* individuals form a single group that did not separate out in the third axis. Vectors 1, 2 and 3 combined contained only 34.3% of the variation indicating that there is little differentiation of the populations (Fig. 3).

Discussion

GENETIC VARIATION

The two populations of *P. diversiflorum* share a high degree of genetic variation and the differentiation of populations, shown in the principal coordinate analysis is minor. There is a tendency for plants from the same population to group together, although the differentiation is based largely on the frequency rather than the uniqueness of RAPD fragments occurring in them and only 34.3% of the variation is accounted for by the first three vectors of the principal coordinate analysis. This suggests that the populations share the same origin. The gene flow estimate of $N_m = 4.2$ indicates that there is, or has been recently, significant gene flow between the populations (Brzosko *et al.* 2002). This is further supported from the partitioning of variation where 88.0% is found within populations. This value is indicative of a species that is highly outcrossing (Hamrick & Godt 1995) and provides another parameter for considering the two populations as two components of a single conservation management unit.

A note of caution must be introduced with regard to inferences based on estimates of gene flow between the Dunkeld and Condah-Hotspur *P. diversiflorum* populations. Although it is possible that gene flow is on-going, mediated by long-distance seed dispersal as reported for other orchid species (e.g. Peakall & Beattie 1991), N_m is a measure

of historical gene flow and does not necessarily represent present-day levels (Brzosko *et al.* 2002). The effects on genetic diversity of rapid and relatively recent fragmentation including extinction of the formerly large population at Gorae West may remain undetected for some time. Hence, the longevity of genets (genetic individuals) is an important consideration.

Morphological characters show extensive variability in *P. diversiflorum*. Nicholls (1942, p. 9) noted: "The (new) species is probably one of the most variable, in regard to floral characters, on record". Rouse (2002) commented that Dunkeld plants showed more variation in floral morphology compared to those at Condah-Hotspur but no published data is available. The genetic data provided by the present study agree with the generally recognised diversity of *P. diversiflorum*.

Terrestrial orchids exist as inconspicuous underground tubers for at least some part of their life cycle (Sydes 1994a, Wong & Sun 1999) and the number of plants emerging can fluctuate widely from year to year. In *P. diversiflorum* populations, plant numbers have been observed to fluctuate remarkably depending on climatic conditions with few plants emerging in particularly dry seasons (Ingeme & Govanstone 1999). The genetic variation detected in Gorae Leek Orchid specimens collected during the 2001/2002 flowering season may represent only a small amount of that present. Many members of the population did not emerge (or produce flowers) owing to the unusually dry conditions, and only 15 plants were sampled per population. Sydes (1994b) reported that in years of drought, abortion of flowers because of water stress occurred in *Thelymitra circumsepta* – an orchid that, similar to *P. diversiflorum*, inhabits swampy areas and flowers during summer. Hence, the proportion of the total genetic variation that is represented by reproductive individuals might change considerably from year to year, depending on how many (and which) plants emerge. Temporal fluctuation in the number of emerging individuals has also been observed in populations of the orchid *Cypripedium calceolus* (Brzosko *et al.* 2002). Temporal variation in emergence may have concealed differences in levels of genetic diversity especially if the dry conditions in 2001 promoted the flowering of certain genotypes and suppressed others. The effect of possible temporal variation and sample size limitations must be taken into account when interpreting the present genetic data. Only long-term monitoring of the populations will resolve this issue.

Numerous authors have reported a significant positive correlation between population size and level of genetic diversity in a variety of plant taxa (e.g. McClenaghan & Beauchamp 1986, Peters *et al.* 1990, Billington 1991, van Treuren *et al.* 1991, Raijmann *et al.* 1994, Godt *et al.* 1996, Sun 1996). Given that the Dunkeld *P. diversiflorum* population is larger in size and contains plants that are more variable in floral morphology than the Condah-Hotspur population (Rouse 2002), it might be expected that the former site also harbours the majority of the species' genetic diversity. Yet our results suggest that the level of genetic diversity is very similar across the two populations (Table 1.). Similarly, Wong and Sun (1999) noted that no such correlation was evident in RAPD data obtained from the endangered terrestrial orchid *Goodyera procera*. However, as was highlighted earlier, it may be too soon to recognize the genetic signature of a recent population bottleneck in *P. diversiflorum*.

MANAGING THE SPECIES FOR CONSERVATION

A primary goal of conservation biology is to ensure the maintenance of biodiversity (Stiling 1999), of which genetic diversity is a fundamental component (Moritz & Faith 1998), and effective conservation programs depend on the identification of unambiguous management units (Avise 1994). Based on findings of the present study, the two extant populations of *P. diversiflorum* can be managed as a single unit, yet site-specific management actions may be required. Fluctuating numbers of emergent plants from year to year do not seem to have had a severe effect on the species' genetic diversity at this stage. This may be due to individuals remaining dormant for several seasons if conditions are not suitable, but subsequently re-emerging and contributing reproductively. Similarly,

Wallace (2002) hypothesized that plant dormancy patterns and chaotic fluctuations in population size from year to year may buffer against the stochastic loss of genetic variation, especially in small populations. Therefore, each year some proportion of the total diversity is cryptic in that it cannot be measured, but generally is not lost. Effective survey is a priority for the management and recovery of rare plants (Hogbin & Peakall 2000). Periodic re-sampling of the two populations for genetic assessment would provide more reliable estimates of the diversity present, would gauge more accurately the extent of population differentiation and importantly, the way in which patterns of genetic diversity can vary from year to year.

The development of propagation techniques should be a high priority of the recovery effort (Rubluo *et al.* 1993) so that an *ex situ* collection can be assembled as a safeguard against the loss of diversity (or of an entire population) at one or both of the sites. This would also allow study of the species' breeding system, and facilitate the production of seed for both supplying *ex situ* collections with seedlings and augmenting seed production *in situ* each season. It would also allow the introduction of *P. diversiflorum* to new sites if considered appropriate, without compromising the continued persistence of the two extant populations. Based on data presented here, the crossing of plants from Dunkeld and Condah-Hotspur populations during the recovery process should be considered as a recovery action given that the long-term risk following the historical loss of populations is that the species has lost some adaptive capacity. In particular, seed produced from within- and between-population crosses will be important for ensuring a broad genetic base for *ex situ* material.

Much of the effort involved in the recovery of *P. diversiflorum* will rely on an understanding of its habitat requirements, population dynamics, breeding system, and life history (Hamrick *et al.*1979, 1991, Ackerman 1998, Hogbin & Peakall 2000). Historically, habitat characteristics for *P. diversiflorum* may be broader than those found at the current sites. If reintroduction and augmentation of populations is undertaken as part of the recovery process, a broad base of genetic variation should promote the establishment of populations with an increased ability to thrive under variable conditions. It is probable that there has been some reduction in the genetic diversity within the species as populations have been lost. However, from this study, the amount of genetic diversity revealed is promising for the long-term survival of the species from an evolutionary perspective. At this stage, both populations must be conserved because despite their similarities they do differ and their small sizes and locations make both vulnerable to loss. Increasing plant numbers by conserving both populations and building an *ex situ* collection can contribute to countering the negative effects of genetic processes associated with small population size (reviewed in Burgman & Lindenmayer 1998).

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