Population diversity of *Doryanthes excelsa* (Doryanthaceae) in eastern Australia

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Abstract: The population diversity of *Doranthes excelsa* Corrêa (Doryanthaceae) was measured from nine distinct geographic populations across eastern Australia, using random amplified polymorphic DNA (RAPD) markers. An UPGMA dendrogram of individuals was derived from squared Euclidian distances based on the Dice (1945) algorithm. Three clusters corresponding to populations at Somersby, Newfoundland and Kremnos Creek populations were found to be distinct from the remainder of the sampled individuals. A Φ_{ST} value of 0.443 indicated that a significant diversity between geographic populations existed; this appeared to be a product of geographical distance and isolation between some of the populations. (PCR = Polymerase Chain Reaction; RAPD = Random Amplified Polymorphic DNA)

The results suggest that there is lesser gene flow between the 'northern' populations (Kremnos Creek and Newfoundland) when compared to the 'southern' populations and that they have a significant level of genetic isolation. The two 'northern' populations should therefore be regarded as being of considerable value for conservation authorities and the commercial breeding sector and should be given priority for conservation. The plants there appear to exhibit a smaller phenotype but confirming this requires further quantification.

Keywords: *Doryanthes*, Doryanthaceae, population, DNA, RAPD, polymorphism.

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Introduction

Doryanthes excelsa Corrêa (family Doryanthaceae), Giant Lily or Gymea Lily, is a spectacular and morphologically distinctive monocotyledon endemic to the open sclerophyll *Eucalyptus* forests of the Central Coast and North Coast of New South Wales, Australia. It is distributed discontinuously from near Wollongong in the south to the hinterland of Coffs Harbour in the north but there as a large gap between the central coast and northern populations. The species develops ensiform leaves to a height of approximately 2 m and inflorescences up to 4 m tall, holding up to 150 large, red flowers (Newman 1928). Because of their size *Doryanthes excelsa* inflorescences are used in large floral displays in hotel foyers, churches, airports and shopping centres (Burchett et al. 1989).

Doryanthes excelsa is highly prized domestically by the Australian floricultural industry and has considerable economic potential as a high-value, cut-flower export crop (Smith 2000). It is also increasingly sought as a landscape plant. At present, many of these cut-stems are sourced from wild populations under licence, or from a very limited number of small-scale plantations. The combination of its high-value and limited supply has led to the illegal harvesting of flowers and theft of seed from wild populations, potentially threatening the long-term sustainability of the species. Urbanisation has also resulted in the destruction of populations.

In order to promote a sustainable cut-flower industry for this species, and to conserve existing wild populations, a novel micropropagation system using immature inflorescences as the explant source has been developed (Dimech *et al.* 2007). The ability to initiate both shoot organogenesis and callogenesis in *Doryanthes excelsa* will form the basis of a viable and environmentally-sustainable industry in the foreseeable future. The development of improved cultivars with desirable commercial traits could add significant value to the crop; molecular breeding programs are likely be reliant upon the identification of novel genes from wild populations.

Given this, there is both a conservation and commercial purpose to measuring the population diversity of this species. Initially, locating sources of variability within wild populations would improve basic understanding of the source and distribution of genetic variability (and potential novel traits) where no research has been performed previously. The aims of this study were to measure the molecular diversity of *Doryanthes excelsa* individuals from nine distinct geographic populations and derive a value of genetic differentiation for wild populations; determine the conservation value of two isolated northern populations and identify sources of diversity that may be useful for future breeding programs.

Materials and Methods

Collection of plant material and sampling procedure

Leaf samples from 87 *Doryanthes excelsa* plants were collected from nine locations (i.e. populations) across the natural range of the species in New South Wales (Table 1, Figure 1). At each location a linear transect was established and plants sampled approximately every 4 m. The intention was to sample ten plants at each site but only eight samples were obtained at Somersby and 9 at Nelsons Bay. As an "out-group", a leaf sample of *Doryanthes palmeri* was collected from a single specimen of unknown provenance growing at the Royal Botanic Gardens Melbourne. Elevation, longitude and latitude were recorded using a global positioning system. Samples were collected in July 2003. In all cases, immature and semi-chlorophyllous leaf material was harvested from the centres of leaf whorls, and placed in resealable polyurethane bags and stored at 4°C in the field, then put into long-term storage at –70°C within a week of collection. Leaf and flower specimens were lodged with the National Herbarium of Victoria (Melbourne).

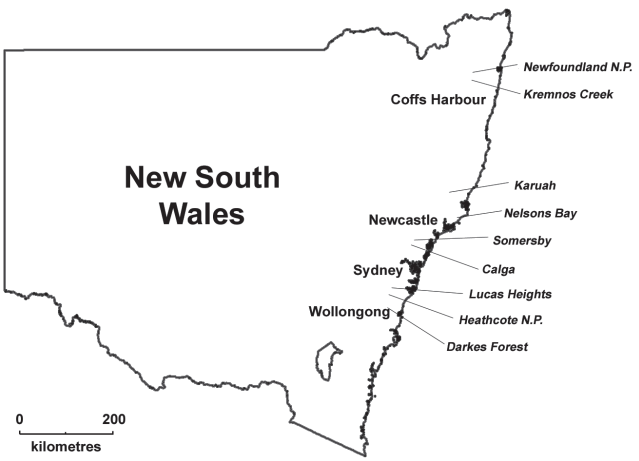

Fig. 1. Collection sites for Doryanthes excelsa populations used in this study.

Extraction of DNA

Frozen leaf material was ground into a powder under liquid nitrogen and the remaining fibres were removed manually. Total genomic DNA was extracted from 20–30 ng of powdered tissue using the Dneasy® Plant Mini Kit (Qiagen, Australia) following the full procedure as detailed in the handbook (January 2004 edition). DNA yield and quality was comparatively quantified with 100 ng µl[1] *Escherichia coli* DNA standards in 1.4% (w/v) agarose gels. Final working solutions of 20 ng μl^{-1} were prepared by diluting concentrated *D. excelsa* DNA stocks in distilled, deionised water. Samples were stored at -20°C.

RAPD PCR Amplification

DNA fragments were amplified using 10-mer oligonucleotide primers (Sigma-Genosys, Australia) selected from the 'Operon' series. Sixty primers were screened for *D. excelsa* and the 10 most informative were used across the entire population, these being A1 (5'-CAGGCCCTTC), A20 (5'-GTTGCGATCC-3'), B8 (5'-GTCCACACGG-3'), B10 (5'-CTGCTGGGAC-3'), C9 (5'-CTCACCGTCC-3'), C11 (5'-AAAGCTGCGG-3'), E1 (5'-CCCAAGGTCC-3'), H8 (5'-GAAACACCCC-3'), J6 (5'-TCGTTCCGCA-3') and K15 (5'-GAGCGTCGAA-3').

Table 1. *Doryanthes excelsa* **populations sampled (in geographic order from north to south) and the number of samples (n) of each. Population distances are calculated using the Haversine Formula (Sinott 1984).**

Population	Code	*n*	Longitude	Latitude	Elevation (m)	Distance to nearest population (km)
Newfoundland	H	10	153° 06'	29° 53'	140	17
Kremnos Creek	E	10	152° 58'	29° 59'	86	17
Karuah	D	10	152° 00'	32° 38'	43	16
Nelsons Bay	G	9	152° 07'	32° 44'	43	16
Somersby	J	8	151° 15'	33° 22'	163	5
Calga	A	10	151°13'	33° 24'	211	5
Lucas Heights	F	10	150° 59'	34° 02'	159	3
Heathcote N.P.	C	10	150° 59'	34° 04'	152	3
Darkes Forest	B	10	150° 56'	34° 14'	380	19

Polymerase chain reaction (PCR) was performed in a volume of 25 µl containing a total of 50 ng of genomic DNA, 2.5 µl of 10× PCR Buffer (200 mmol l^{-1} of Tris-HCl and 500 mmol l^{-1} KCl, pH 8.4; Invitrogen, Australia), 3.6 mmol l^{-1} of $MgCl_2$, 240 µmol l^{-1} of dNTPs, 0.8 µmol l^{-1} of primer and 1 U (0.2 µl) of *Thermus aquaticus* DNA polymerase (Invitrogen, Australia). The amplification reaction consisted of an initial denaturation step of 94°C for 1 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 45°C for 30 s and extension at 72°C for 1 min. A final extension of 72°C for 5 min completed the reaction. Where individual bands did not reproduce, the data were excluded. All experiments were performed twice to ensure reproducibility (He *et al* 1994).

Amplification products were separated on a 1.4% (w/v) agarose gel for 2 h at 90 V in TAE buffer. The products were stained using SYBR® Green I dye and fragments within the range 350–2700 bp were observed under UV-light (λ=620 nm), and photographed using the Kodak *1D* computer programme

Data analysis

Statistical analyses of RAPD patterns were based on the following assumptions: (*i*) that RAPD fragments were diploid and dominant markers with loci being either present (amplified and scored as '1') or absent (non-amplified and scored as '0'); (*ii*) that the co-migration of fragments indicated identical loci and (*iii*) that polymorphic loci were subject to Mendelian inheritance. RAPD data were manually entered into a binary data file. Genetic distances between individuals were calculated using the Dice (1945) similarity coefficient and were subjected to Unweighted Pair-Grouped Method with Arithmetic Means Analysis (UPGMA) clustering using the *SAS* (SAS Institute, USA) statistical package. An UPGMA dendrogram was derived from Euclidian distances, calculated as [1-Dice coefficient]. A two-dimensional ordination was created using the non-metric multidimensional scaling (NMDS) procedure in *SAS* version 9.1.3 with the default options apart from formula=2 dim=2 and fit=2.

Divergence between populations was estimated as Φ_{ST} and tested by Analysis of Molecular Variance (AMOVA) using *GenAleEx 6.1* (Peakall and Smouse 2006). A two-way AMOVA was used to measure the spread of molecular variance between and within all sampled populations of *Doryanthes excelsa* as well as the 'northern' and 'southern' populations in isolation. A three-way AMOVA was used to indicate the levels of molecular variance at the regional ('northern' and 'southern') and population levels. A spatial structure analysis was performed using *GenAleEx 6.1* (Peakall and Smouse 2006) to test whether isolation was correlated with geographical distance. In all analyses, statistical significance was indicted where $p<0.05$.

Results

Ten RAPD primers produced a total of 103 scorable fragments across 87 individuals. The number of bands produced with each RAPD primer ranged from 5 to 14 and were between 350 bp and 2700 bp in size. The number of polymorphic loci measured across all individuals was 91 (88.4%).

Table 2 a) Three-level Analysis of Molecular Variance (AMOVA) indicating molecular variance amongst and between regions and populations of *Doryanthes excelsa*. df = degrees of freedom; SS = sum of squares; MS = mean of squares.

Source	df	SS	Estimated Variance	Percentage Variance	Φ	Probability
Regions	1	1.031	0.024	18%	0.180	<0.001
Populations within Regions	7	2.068	0.022	17%	0.201	<0.001
Within Populations	78	6.722	0.086	65%	0.345	
Total	86	9.891	0.132			

b) Two-way AMOVA indicating the percentages of molecular variance amongst and between the two 'northern' populations of *Doryanthes excelsa.*

Source	df	SS	MS	Estimated Variance	Percentage Variance	Φ	Probability
Populations	1	0.532	0.532	0.044	32%		
Within Populations	18	1.674	0.093	0.093	68%	0.321	0.000
Total	19	2.206	0.625	0.137			

c) AMOVA indicating the percentages of molecular variance amongst and between the 7 'southern' populations of *Doryanthes excelsa.*

Source	df	SS	MS	Estimated Variance	Percentage Variance	Φ	Probability
Populations	6	1.536	0.256	0.018	18%		
Within Populations	60	5.048	0.084	0.084	82%	0.176	0.000
Total	66	6.584	0.340	0.102			

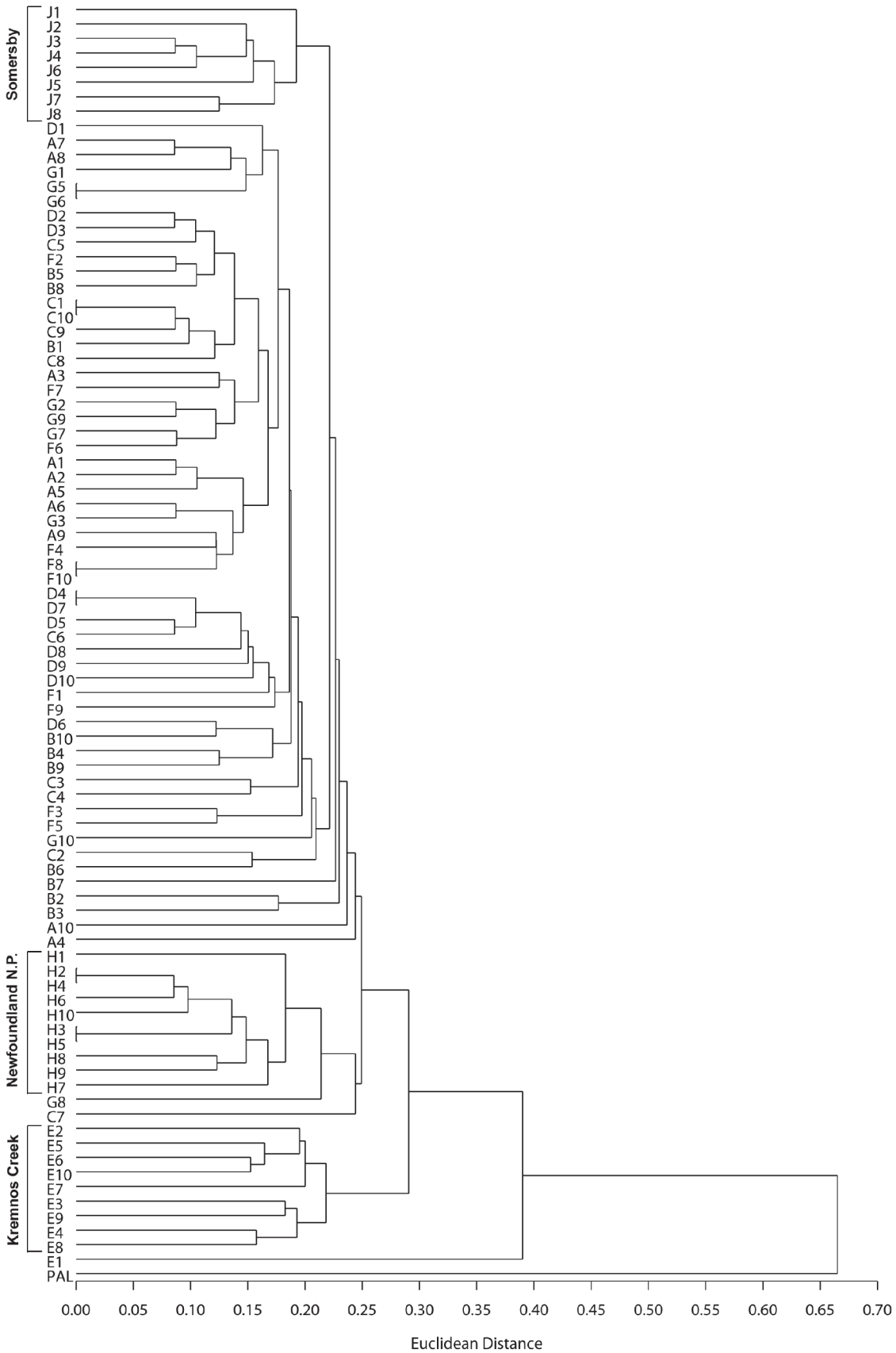

Fig. 2. An UPGMA dendrogram showing the squared Euclidian distances of the individuals from the nine *Doryanthes excelsa* populations. Each individual is labelled with a two-letter prefix indicating the population, and a numeric accession number. A=Calga; B=Darkes Forest; C= Heathocote National Park; D=Karuah; E=Kremnos Creek; F=Lucas Heights; G=Nelsons Bay; H=Newfoundland State Forest; J=Somersby; PAL=*Doryanthes palmeri* (for comparison).

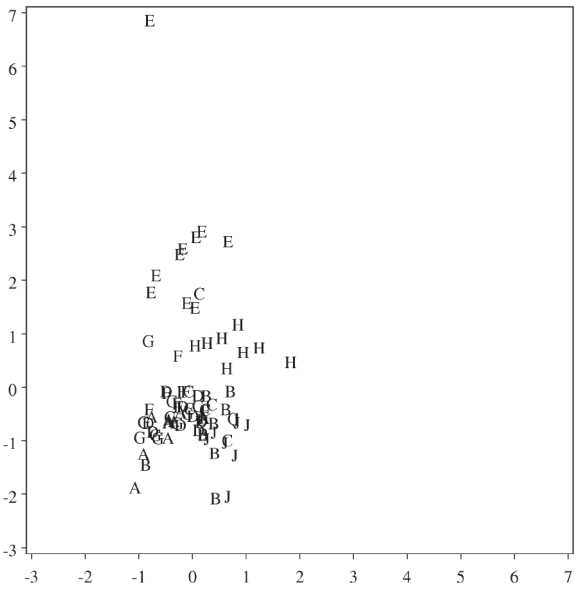

Fig. 3. A two-dimensional NMDS ordination showing the relationship between individuals in nine populations of *D. excelsa*. A=Calga; B=Darkes Forest; C= Heathcote National Park; D=Karuah; E=Kremnos Creek; F=Lucas Heights; G=Nelsons Bay; H=Newfoundland State Forest; J=Somersby. The distance stress$_2$ = 0.165.

The greatest genetic distance between any two individuals was 0.044 for E1/J1 as well as B3/J8. The genetic distance of 0.00 between individuals was found between D4/D7; G5/G6; C1/C10; F8/F10; H3/H4 and H2/H5, indicating that these individuals may be clones.

An UPGMA dendrogram (Figure 2) shows three clusters corresponding to the Somersby (J) and Newfoundland (H) populations and 9 of the 10 Kremnos Creek (E) individuals. The remaining Kremnos Creek individual (E1) was distinct from all other samples including *Doryanthes palmeri* (PAL), though it was closest to the other Kremnos Creek individuals. The remainder of individuals and populations did not obviously cluster according to geographic distribution.

A two-dimensional NMDS ordination (Figure 3) demonstrated the diversity of *Doryanthes excelsa* across the nine populations (*Doryanthes palmeri* was not included because the large distance between it and the *D. excelsa* individuals). The picture is very similar to that shown by cluster analysis: the Newfoundland State Forest (H) and Kremnos Creek (E) populations were distinct and the Somersby (J) population lay slightly to the right of the remaining individuals but was overlapping with the other central coast populations.

A two-level analysis of molecular variance (AMOVA) indicated that there was a highly significant divergence between individuals of different populations, with a recorded Φ_{ST} of 0.443. When the individuals from the 'northern' populations at Kremnos Creek and Newfoundland State Forest were excluded, Φ_{ST} was reduced to 0.297 but remained highly significant.

A three-level AMOVA was used to measure the percentage of molecular variance amongst regions, populations and within populations where the Kremnos Creek and Newfoundland populations were categorised as belonging to a 'northern' region, and the remaining populations to a 'southern' region. A value of Φ was calculated for each level; it was found that 65% of the variation lay within populations, 18% amongst regions and 17% amongst populations within regions (Table 2a).

A two way ANOVA on individuals from only the two 'northern' populations (Kremnos Creek and Newfoundland), showed 68% of the variation lay within populations and 32% amongst the populations (Table 2b). A two way ANOVA on individuals from the seven 'southern' populations (Somersby, Karuah, Calga, Nelsons Bay, Heathcote N.P., Lucas Heights and Darkes Forest) showed 82% of the variation lay within populations and 18% amongst the populations (Table 2c).

Discussion

RAPD markers showed measurable genetic differences within and between geographical populations of *Doryanthes excelsa.* A high degree of genetic differentiation was found between individuals of different populations, compared to individuals within populations. Overall, Φ_{ST} was 0.443, suggesting long isolation between populations. This value is higher than equivalent estimates for 'comparable' genera such as *Iris* and *Lilium*, when a comparison was made between Φ_{ST} and Nei's G_{ST} (Nei 1972) for related species. Arafeh *et al.* (2002) used RAPD data to calculate a G_{ST} of 0.21 for 242 individuals of *Iris atrofusca* and *Iris haynei* from Israel and Palestine indicating lower among-population differentiation than in *Doryanthes excelsa*. They also found greater within-population similarity in *Iris atrofusca*/*Iris haynei* than in *Doryanthes excelsa*, as is expected given that clonal reproduction in *Iris* is widespread, unlike *Doryanthes*.

In the UPGMA dendrogram, individuals outside the 'northern' populations (Kremnos Creek and Newfoundland) or Somersby (a 'southern' population) formed a loose cluster which suggests relative genetic similarity. These populations were in relatively close geographic proximity and so it may be expected that interpopulation diversity would be low relative to intrapopulation diversity. The proportion of molecular variance within the seven 'southern' populations (82% , Φ_{IS}=0.176) (Table 2c), supports the hypothesis that gene flow is relatively widespread between these populations as a whole.

The separation of the 'northern' populations (Kremnos Creek and Newfoundland) was likely due to a limitation in gene flow arising from geographical isolation. A distance of 330 km separates the northernmost Central Coast population

of Nelsons Bay and the Newfoundland population. Since birds represent the most common vector for sticky pollen transfer in *Doryanthes*, one might expect minimal bird activity between these populations with gene flow reduced as a result,. According to Nash (1996) *Doryanthes excelsa* is pollinated by Cockatoos (*Cacatua galerita*) and Pied Currawongs (*Strepera graculina*) as well as smaller bird species that could carry pollen between the populations.

Whether or not recent gene flow has occurred between the 'northern' and 'southern' populations, the use of allele-specific markers would permit the measurement of allele type frequencies within each population. If such long-distance pollination occurred on even an occasional basis (an idea supported in part by the Φ_{ST} value and AMOVA calculations), allopatry could still be prevented. However the effectiveness of pollination of *Doryanthes excelsa* by large birds is limited because of the damage these species do to the flowers (Nash 1996).

The sampled individuals from Somersby also showed a relatively high degree of genetic differentiation, though unlike the 'northern' populations, Somersby was in close proximity to the other 'southern' populations. For instance, the Somersby and Calga populations were almost adjacent, separated by only 5 km across Brisbane Water National Park. The Calga site, substantially cleared and used for light agriculture for a considerable period has had greater agricultural development than the Somersby site, which was primarily composed of lightly cleared open forest with intermittent development.

The Calga plants were generally limited to a long, narrow tract of land between a farm fence and a road and it could be hypothesised that the Calga population is experiencing a bottleneck owing to a much-reduced population size and restricted gene flow due to the linear arrangement of surviving individuals. A bottleneck effect has been observed in other taxa, e.g. in Taiwan Wen and Hsiao (2001) found greater variation within high-altitude populations of *Lilium longiflorum* var. *formosanum* (Liliaceae) compared to the lower altitude populations, thought to be attributable to the diminished population size of the low-altitude populations as a result of agricultural expansion. In the predominantly outbreeding (Newman 1929) *Doryanthes excelsa*, there are generation times that could counteract bottlenecks and maintain intrapopulation diversity. Persson *et al.* (1998) found this to be the case for *Lilium martagon* (Liliaceae) growing in Europe.

The longevity of individual plants can ensure the preservation of alleles, even in small populations. *Doryanthes excelsa* may take up to 10 years from germination to flowering, and will live for decades. Most individuals will survive fires (the most common and dramatic disturbance event in their ecosystem) by resprouting after the forest has burnt (Denham & Auld 2002). The longevity of individuals means that the complete extinction of alleles within a population may be delayed for decades, and where there have been multiple selective events in succession, important alleles may be preserved throughout the period. However, commercial and/or illegal harvesting of inflorescences jeopardises this process and makes the population more vulnerable by reducing the capacity of individual plants to reproduce in any one season. The use of a co-dominant marker system in a further investigation of population diversity would shed further light on this issue. A phenotypic assessment would also be valuable to map any provenance types that may provide commercial opportunities for the cut-flower industry.

The results of this study suggest that there is lesser gene flow between the Kremnos Creek and Newfoundland populations, when compared to the 'southern' populations and that they have a significant level of genetic isolation. The two 'northern' populations should therefore be regarded as being of considerable value for both conservation authorities and the commercial breeding sector and should be given priority for conservation for both environmental and commercial reasons. The plants there appear to exhibit a smaller phenotype but confirming this requires further quantification.

By identifying the centres of molecular diversity, this study is an important first step towards developing *Doryanthes excelsa* as a horticultural resource. Through this process, this study provides an important basis upon which future conservation programs should be based, ensuring that wild populations remain in abundant numbers and that the Australian cut-flower industry can benefit from the development of this novel product.

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