

Population genetic structuring in a rare tropical plant: *Idiospermum australiense* (Diels) S.T. Blake

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Abstract *Idiospermum australiense* (Diels) S.T. Blake is considered to be one of the few remaining species of an ancient assemblage to have survived the attrition of Australian tropical rain forest during historically drier periods. This monotypic species is currently restricted to two very wet lowland rain forest locations in Australia's wet tropical World Heritage Area that are thought to have provided refuge for humid-adapted taxa during the last glacial maximum. Two dominant (RAPD and ISSR) molecular marker sets were employed to investigate whether the genetic structure of *Idiospermum* could be attributed to its restriction to these quite disjunct localities. The results reveal that neither its restriction to purported Pleistocene refugia nor the geographic distance between populations could fully explain the distribution of variation in the *Idiospermum* data set, with evidence to suggest that potentially deeper time events have played a role in population structuring and the distribution of diversity. Although there is sufficient evidence in the data to suggest that gene dispersal is quite limited in the species, further investigation is still needed to yield more informative detail on additional factors, such as breeding and germination strategies and their potential influence over population structuring and diversity levels within each population and refugium.

Keywords *Idiospermum* · RAPD · ISSR · Population structure · Lowland tropical · Rain forests

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Introduction

The monotypic *Idiospermum australiense* (Diels) S.T. Blake (idiot fruit) presents a unique opportunity to investigate historical biogeographical relationships in the Australian Wet Tropics. It is a long-lived tree with a possible Campanian (Upper Cretaceous) divergence from its sister genera in the Calycanthaceae and represents a lineage of considerable antiquity in the angiosperms (Zhou et al. 2006). It is viewed as one of the few remaining species of an ancient assemblage to have survived the attrition of rain forest during historically drier periods (Goosem 2002), periods that include the drying of the Australian continent as a result of the break up of Gondwana and the extreme climatic fluctuations of the late Pleistocene. *Idiospermum* has the heaviest diaspores (70–235 g, mean approximately 130 g) of any dicotyledonous flowering plant in Australia (Edwards et al. 2001) and is restricted to two very wet lowland rain forest locations in Australia's Wet Tropics (Fig. 1) below 200 m elevation (Worboys 1999).

The aridification of the Australian landscape has resulted in the marginalisation and fragmentation of the Australian wet tropical rain forests (Webb and Tracy 1981; House and Moritz 1991; Schneider and Moritz 1999). The two areas where *Idiospermum* is found are both purported to have provided significant refuge for humid-adapted species especially during the last glacial maximum approximately 18,000 years ago (Hugall et al. 2002). These refugia are separated by approximately 130 km.

In this paper the term 'refugia' (northern and southern 'refugium') represent what Worboys (1999, 2003) referred to as northern and southern populations with the term 'populations' used in reference to the discrete sites where *Idiospermum* has been recorded within each refugium. In the southern refugium *Idiospermum* has only been

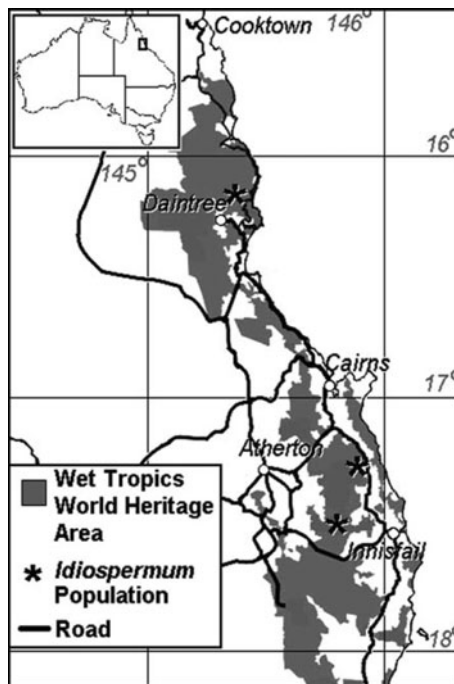


Fig. 1 Distribution of *Idiospermum australiense* within the Australian Wet Tropics

recorded within the catchments of Harvey Creek near Mt Bellenden Ker and Russell River near Mount Bartle Frere, with only one quite discrete and aggregated population of individuals in each of these catchments (Worboys 1999). Across the northern refugium *Idiospermum* has been recorded within the catchments of Cooper, Hutchinson, Oliver and Noah Creek near Cape Tribulation, and although representing a smaller potential distributional area compared to the southern refugium, individuals and populations of individuals are more abundant in this refugium with at least nine populations recorded (Worboys 1999).

In this study two molecular marker systems—random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers—were utilized to investigate genetic structure among and between populations and refugia. If distance is the major barrier to gene flow, genetic differentiation between population pairs will follow an isolation by distance (IBD) model and genetic diversity will be lower in more isolated populations.

Materials and methods

Population sampling and DNA extraction

Leaf material was collected from nine individuals in each of two populations from each refugium (Fig. 1): two populations, at a distance of approximately 2 km apart, from

the Cooper Creek catchment in the northern refugium (Hewitt's Property 16°10', 145°25'; Turpentine Road 16°10', 145°25'); and the two populations, separated by a distance of approximately 25 km, in the southern refugium (Russell River 17°27', 145°47'; Harvey Creek 17°15', 145°54').

DNA was extracted from silica-dried leaf material using a modified hot cetyltrimethyl ammonium bromide (CTAB) extraction method (Doyle and Doyle 1987). DNA templates were quantified using a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia Biotech).

RAPD and ISSR protocol

Four RAPD primers (Operon Technologies) and two ISSR primers inversely anchored at the 3'-end (Wolfe et al. 1998) were selected for amplification (Table 1). Each 25 μ L RAPD and ISSR reaction contained 25 ng of template DNA, 0.4 μ M of each primer, 0.5 units of *Taq* DNA polymerase, recombinant (Invitrogen Life Technologies), 200 μ M each dNTP (Promega), 4 ng of bovine serum albumin (Sigma-Aldrich) and 1 \times *Taq* buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl] (Invitrogen Life Technologies). RAPD reactions contained 2 mM MgCl₂ (Invitrogen Life Technologies). ISSR reactions contained 3 mM MgCl₂ (Invitrogen Life Technologies).

RAPD- and ISSR-PCR amplifications were carried out in Omn-E thermocyclers (Hybaid). Prior to the *Taq* DNA polymerase being added, RAPD and ISSR reactions were heated to 60°C for 30 min to allow for the BSA to bind to proteins and remove them from the reaction. RAPD-PCR reactions were submitted to an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 36°C and 2 min at 72°C. This was followed by a final extension step of 2 min at 72°C. ISSR-PCR reactions were submitted to an initial denaturation cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. This was followed by a final extension step of 10 min at 72°C. Reproducibility of results was confirmed by running repeat reactions with each assay, along with negative controls.

Table 1 RAPD and ISSR primers used in the molecular analysis of *Idiospermum australiense*

Primer	Sequence 5'–3'
OPC-08	TGGACCGGTG
OPB-02	TGATCCCTGG
OPB-05	TGCGCCCTTC
OPB-07	GGTGACGCAG
ISSR-857	(AC) ₈ YG ^a
ISSR-17878B	(CA) ₆ GT

^a Y = C/T

Amplification products were identified by electrophoresis on 2% (w/v) agarose gels in $1.0\times$ TBE buffer and visualised with ethidium bromide (4 ng/mL) under UV light. Gels were run for 3 h at 70 V after an initial 10 min run at 100 V to move products from the sample wells. A 1 kb ladder (Northern Biotech) was included on each gel in order to correctly identify each scorable locus between gels.

Data analysis

PCR products were scored at each RAPD and ISSR locus as either present (1) or absent (0) for each fragment for all individuals. Scoring of RAPD and ISSR bands is limited to binary characters due to the dominant nature of these markers (Nybom and Bartish 2000; Nybom 2004; Bussell et al. 2005). Because there are a number of factors that can influence fragment yields (Nybom 2004; Bussell et al. 2005) only loci that could be unambiguously scored as present or absent regardless of band intensity were included in the analysis.

GenAlEx 6: genetic analysis in Excel software (Peakal and Smouse 2005) and Arlequin ver 2.000 genetic data analysis software (Schnieder et al. 2000) were used to perform an analysis of molecular variance (AMOVA) to investigate how the variation in distances between molecular phenotypes was hierarchically distributed within and among populations, and refugia. Based on recommendations of Kosman and Leonard (2005) for dominant markers, a squared Euclidean distance (also known as the simple mismatch coefficient) was used to estimate phenotypic distances between individuals. In conjunction with the AMOVA, the GenAlEx software also provided Φ_{PR} (the proportion of variance among populations within refugia, relative to the variance among and within populations), Φ_{RT} (the proportion of the variance among refugia, relative to the total variance) and Φ_{PT} (the proportion of the variance among populations, relative to the total variance) (Peakal and Smouse 2005). Φ_{PT} is an analogue to F_{st} when used with haploid or binary data (Peakal and Smouse 2005). As a measure of pair-wise population differentiation, Φ_{PT} values were also calculated for each possible population pairing. All Φ values were tested for their significance against null distributions generated from random permutations of the data set (999 plus the original data set).

Mantel tests were used to investigate whether variation in the data set could be correlated with geographic distance. Mantel tests are employed to look for correlations between non-parametric distance matrices (McCune and Grace 2002) and in this study provided a correlation value (Mantel r) of pair-wise population subdivision (Φ_{PT}) over pair-wise population geographic distance. Analysis was

performed using GenAlEx V5 software. The correlation value was tested for significance against a null distribution generated from random permutations of the data sets (999 plus the original data set).

Band diversity as an estimate of genetic diversity within each population was established using the Shannon's diversity index (S , as denoted by Allnutt et al. 1999). POPGENE (ver 1.31) genetic data analysis software (Yeh et al. 1999) was employed to calculate the S values. In this analysis each band is treated as a single locus (Allnutt et al. 1999; Yeh et al. 1999; Bekessy et al. 2002). Mean values among populations and between refugia were compared via an analysis of variance (ANOVA) of transformed data.

Results

A total of 36 loci were scored across all individuals (22 RAPD and 14 ISSR); 16 (44.44%; 9 RAPD and 7 ISSR) were polymorphic. Only these polymorphic loci were used to construct squared Euclidean distance matrices and for the calculation of Shannon's diversity Indices.

Population structure

Over 50% of the variation in the data set could be attributed to within population differences with a significant Φ_{PT} of 0.46 (Table 2). The remainder was equally apportioned to among populations within refugia (23%, Φ_{PR} 0.302) and among refugia (23% Φ_{RT} 0.226) (Table 2).

There was a highly significant difference between all pairs of populations (Φ_{PT}) (Fig. 2). The level of subdivision between the Harvey Creek and Russell River populations fell within the range of values recorded for the most geographically distant pairs (Fig. 2). There was no significant correlation between pair-wise population Φ_{PT} values and geographic separation ($r = 0.648$, $p = 0.082$).

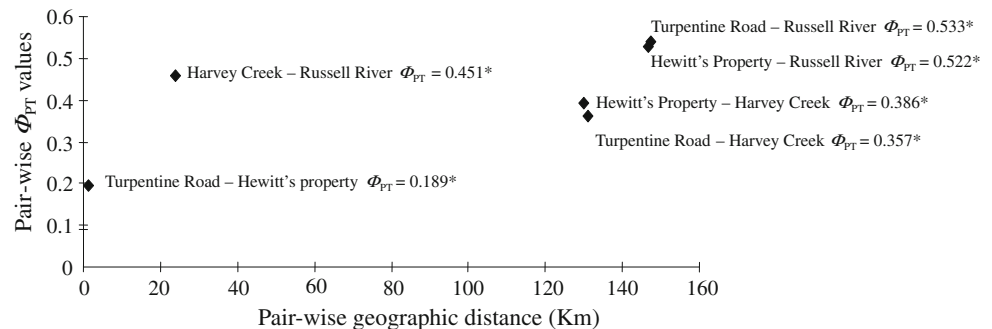
Levels of diversity

Three out of the four populations contained two or more individuals with identical banding profiles (Table 3), but no molecular phenotypes were shared between populations. Consistently, the proportion of individuals with distinguishable phenotypes and the percent variable bands were lowest in the two southern populations (Table 3).

To investigate the probability that phenotypes represented unique genets in each of the three populations that contained individuals with identical banding profiles, the observed probability of the combined phenotypes occurring within each population was compared to a null

Table 2 An analysis of molecular variance (AMOVA) conducted on nine individuals from each of four populations of *Idiospermum australiense* using RAPD and ISSR phenotypes (bi-refugial structure)

Source of variation	df	SSD	MS	Variance component	Value	Percentage of variance
Among refugia	1	20.278	20.278	0.682	0.226* (Φ_{RT})	23
Among populations within refugia	2	16.000	8.000	0.708	0.302* (Φ_{PR})	23
Individuals within populations	32	52.222	1.632	1.632	0.460* (Φ_{PT})	54

* $p < 0.001$ **Fig. 2** Mantel correlation test of pair-wise population Φ_{PT} values over geographic distance conducted on *Idiospermum australiense*. Asterisks indicate Φ_{PT} values with a p value of <0.001 **Table 3** The diversity in banding patterns of combined RAPD and ISSR loci within populations of *Idiospermum australiense*

Population	No. of plants examined	No. of banding profiles	Proportion of distinguishable phenotypes (PD)	Variable bands (%)
Hewitt's Property	9	9	1.00	25.00
Turpentine Road	9	8	0.89	25.00
Harvey Creek	9	6	0.67	11.11
Russell River	9	7	0.78	13.89

probability distribution constructed for each corresponding population. This model assumes that loci are independent and that sampling is random. *Idiospermum* is a facultatively outcrossing species with no evidence of self-fertilisation during pollinator exclusion trials (Worboys and Jackes 2005). Firstly, a frequency distribution of possible molecular phenotypes was randomly generated (10,000 permutations) for each population based on the frequency of the presence and absence of each variable band in each population data set. A null probability distribution was then generated by randomly selecting nine possible phenotypic combinations (10,000 permutations). The probability of their combined frequencies formed the basis of the null distribution to which the observed probability of the combined molecular phenotypes was compared. If the observed probability fell within the 95% confidence interval (CI) then sampling was considered random. If the observed probability fell outside the lower end of the 95% CI this was considered suggestive of the presence and influence of potential clones over the observed frequency of molecular phenotypes. Only one

population, Harvey Creek, which had the highest proportion of individuals with identical phenotypes, yielded a significant difference from the null distribution (observed $p = 3.04 \times 10^{-14}$; 95% CI = 1.23×10^{-12} to 6.69×10^{-13}). The distributions in the three remaining populations were consistent with random sampling.

Figure 3 presents mean band diversity within each of the four populations using the Shannon's diversity index (S). The lowest values were recorded for the southern populations (Harvey Creek 0.186 ± 0.286 , Russell River 0.177 ± 0.281) compared to the northern populations (Hewitt's Property 0.334 ± 0.287 , Turpentine Road 0.362 ± 0.340). The Turpentine Road population, which contained two individuals with shared banding profiles, had the highest mean value of the four surveyed. Transformed S values for the four data sets were not statistically different from one another ($F = 1.675$, $p \geq 0.05$). However, when grouped by refugia there was a significant difference ($F = 5.144$, $p = 0.027$): diversity was lower in the southern refugium (south 0.181 ± 0.279 SD versus north 0.348 ± 0.310 SD) (Fig. 4).

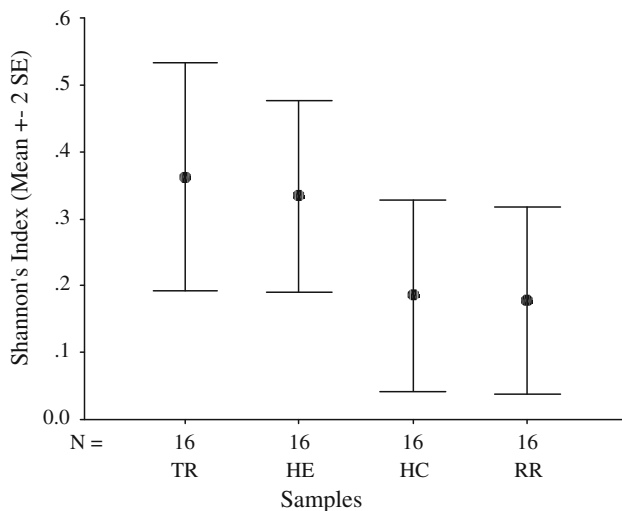


Fig. 3 Mean estimates of genetic diversity ($\pm 2SE$) within populations of *Idiospermum australiense* using Shannon's diversity indices (S). *TR* Turpentine Road, *HE* Hewitt's Property, *HC* Harvey Creek, *RR* Russell River

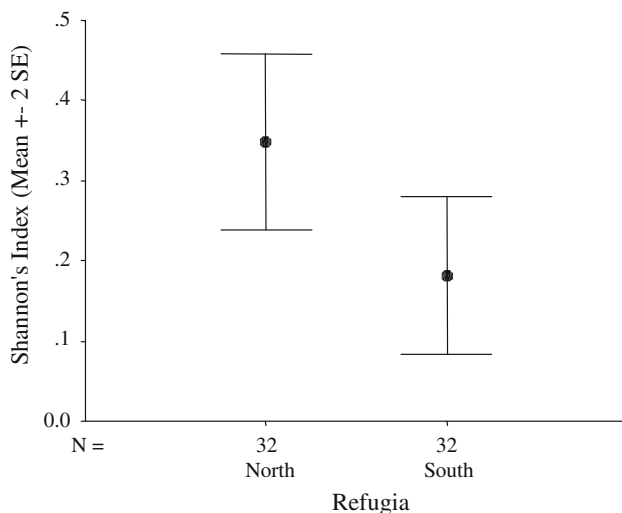


Fig. 4 Mean estimates of genetic diversity ($\pm 2SE$) grouped by refugia containing populations of *Idiospermum australiense* using the Shannon's diversity index (S). *North* Hewitt's Property and Turpentine Road, *South* Harvey Creek and Russell River

Discussion

Population structure

Structure is clearly evident across the four populations of *Idiospermum* surveyed. However, the distribution of variance could not be solely attributed to a contraction and division in the distribution of *I. australiense* caused by the last glacial maximum. While a significant proportion of variance could be accredited to the physical disjunction between refugia, within each there was significant differentiation. This indicates that the severity of a Pleistocene-

related bottleneck was not sufficient to homogenise and fix alleles within refugia.

Nor could structure be attributed to the geographic distance between populations. If distance were the only barrier to influence rates of gene flow then the average phenotypic distance (or the level of phenotypic differentiation) between populations would increase monotonically as the geographic distance between pairs increased, i.e., the IBD model (Sork et al. 1999; Tero et al. 2003). The two populations in the northern refugia are genetically distinct, despite their physical proximity. Significant differentiation between the two northern populations over such a relatively small distance indicates a limited ability for gene dispersal in this species. This would be consistent with the hypothesis that gravity constitutes the primary mode of fruit dispersal (Worboys 1999). No contemporary biotic dispersal vector has been clearly identified for *Idiospermum*'s heavy diaspores, which are also highly toxic (Duke et al. 1995). It also suggests that there are major constraints on pollen dispersal in the species. Small beetles and thrips have been identified as the most likely potential pollinators (Worboys and Jackes 2005). The small size and limited vagility of these insects alone may infer an inability of the diaspores to disperse far beyond population limits.

The two southern populations are also genetically distinct. The level of subdivision (Φ_{PT} value) uncovered between these populations could indicate that the two mountain ridges (1,211 and 1,615 m respectively) that separate them have acted as significant barriers to dispersal. These peaks (or batholiths) are composed of granite extruded 250 to 235 million years ago (Willmott and Stephenson 1989). Subsequent differential weathering and erosion has led to the vertical dominance over the North Queensland Highlands of these more resilient granite protrusions (Willmott and Stephenson 1989; Nott 2003). Given the relative antiquity of the *Idiospermum* lineage, the possibility exists that the influence of these mountain peaks over population structure predates the last glacial maximum.

Genetic diversity

Genetic studies conducted on the endangered shrub *Haloragodendron lucasii* (Sydes and Peakall 1998) and the rare herbaceous grass *Calamagrostis porteri* ssp. *insperata* (Esselman et al. 1999) found that diversity within populations was positively correlated with the number of unique genotypes and phenotypes identified. Comparatively lower band diversities, although not statistically significant, were also found to be more closely associated with the two populations of *Idiospermum* (Harvey Creek and Russell River) containing the highest proportion of individuals with identical banding profiles. In one southern population (Harvey Creek), the probability of drawing the number of

identical phenotypes identified was significantly lower than if sampling was random. *Idiospermum* has a remarkable germination potential. Embryos contain two to six cotyledons and when separated, each cotyledon can produce a seedling (Edwards et al. 2001). Therefore, it is possible for two or more clones to establish, which would reduce genetic diversity within populations. However, it is also important to stress that studies employing dominant markers to identify genotypes are confounded by the inability of these markers to identify alternative alleles at each locus. As a consequence, the banding profiles are likely to provide a conservative estimate of genetic diversity, but the relative differences may still be valid.

When grouped by refugia there was a significant difference in diversity estimates, which may reflect the influence exerted by the lower numbers of unique molecular phenotypes (potential clones) identified in the southern populations. However, limited discriminative and statistical power, differences in census sizes, differences in the distances and barriers between populations and a possible influence from a recorded difference in floral anatomy between refugia (Worboys 2003) over diversity levels lends ambiguity to this interpretation. Worboys (2003) has suggested that the difference in floral anatomy may reflect a divergence among lineages between refugia as a consequence of reproductive isolation.

Census sizes are smaller in the south than those in the north. Although more accurate counts are not available at this time, S. Worboys (pers. comm.) estimates that there are no more than 50 mature individuals in each of the southern populations compared to hundreds of individuals in the northern distribution. Empirical evidence suggests that with decreasing effective population size, the influences of random genetic drift and inbreeding over genetic variability increase (Frankham 1996; Frankham et al. 2004). Smaller populations are more likely to have lower genetic diversity than larger populations (Frankham 1996; Frankham et al. 2004).

In contrast to the case in the south, the closer proximity of northern populations to each other and potentially weaker barriers to gene flow between these populations could translate to higher dispersal frequencies, at least within the Coopers Creek catchment. Another population located in an adjacent catchment area (Oliver and Noah Creek) separated by a low peak (424 m) from the Coopers Creek catchment was not screened in this study. The differences in floral assemblages between the refugia identified by Worboys (2003), which translate to higher pollen-to-ovule ratios in the northern refugium may convey higher outcrossing rates upon these populations. Combined, these factors could mean that the northern refugium has been historically better able to support higher levels of genetic diversity and maintain higher gene flow than its

southern counterpart despite the tendency of clonal reproduction to reduce genetic diversity.

Conclusion

The differentiation among the southern populations indicates that historically gene flow has been limited between the two. Given the small census sizes in the southern refugium, differentiation may have been intensified by genetic drift. The differentiation among the northern populations over such a relatively small geographic distance and in the absence of a clear dispersal barrier indicates that dispersal is limited. The weight and toxicity of the diaspores and absence of any evidence of biotic vectors strongly suggests that dispersal is limited to physical processes.

The results are concordant with previous population genetic studies conducted on a range of fauna endemic to the Australian Wet Tropics and the one floral study (Pye and Gadek 2004) conducted on the more widespread Australian conifer *Araucaria bidwillii* (Bunya Pine). Most reveal a genetic signature that can be related to the impact of the late Pleistocene climate, but several conclude that vicariant events preceeding this time have also exerted a strong influence over genetic structure (e.g. Schneider et al. 1998; Bell et al. 2004; Pye and Gadek 2004). However, as Pye and Gadek (2004) caution, the impact of the last glacial maximum over genetic structure may not yet be fully realised in species with long generational times, most of which are woody plant species like *Idiospermum*. Species with long generational times are considered to hold stronger historical genetic signals due to slower rates of genetic decay than short-lived species (Loveless and Hamrick 1984; Dutech et al. 2003). This would mean that the genetic structure and diversity among upcoming generations of *I. australiense* could reveal even greater disjunction and allelic depauperation.

Future research

Future actions would include the addition of larger sample sizes, numbers of variable loci and samples, and the development and supplement of co-dominant genetic markers. Larger sample sizes and numbers of loci will ensure that a greater sample of variation is captured, and with the inclusion of greater population numbers, will allow for a more complete picture of the genetic structure of *I. australiense*. A greater understanding of the mating, dispersal and germination dynamics of the 'idiot fruit' tree would further facilitate the conservation and management of this rare endemic species from Australia's northern tropical rain forests.

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