Analysis of genetic variation in a disjunct, narcotic producing, population of *Duboisia hopwoodii* (F. Muell.) F. Muell.

P. French, E. James and N. Walsh

National Herbarium of Victoria, Royal Botanic Gardens Melbourne, Birdwood Avenue, South Yarra Victoria 3141, Australia; email: p.french2@student.unimelb.edu.au

Introduction

The native Australian narcotic 'Pituri' was used by Aboriginal people of western Queensland as a social lubricant and an enhancer of endurance and strength. At the time of European settlement the trade of Pituri was extensive, covering an area of 550 000 square kilometres (Watson 1983). Pituri is obtained from the leaves and small twigs of the plant *Duboisia hopwoodii* (F. Muell.) F. Muell., family Solanaceae (Figure 1). The leaves were dried and mixed with ash to make a quid for chewing (Keogh 2011). H. Beckler, who tried the drug on the Burke and Wills expedition in 1861, described it as producing "a dreamy voluptuous sensation" (Roth 1897:31). By coincidence, Beckler collected the type specimen of *D. hopwoodii* on this same expedition, unaware that it was the source of the drug Pituri. *Duboisia hopwoodii* is an arborescent shrub which grows to 3 m tall and occurs across the arid zone of Australia (Maiden 1893). However the most highly sought after form of this potent drug has been obtained from an isolated population in the north east of the Simpson Desert in the Mulligan River region (Figure 2). The plants in this area produce higher levels of the narcotic nicotine and lower levels of the highly toxic substance D-nor-nicotine relative to plants occurring in other areas (Barnard 1952). The exact location of this unique population was once a closely guarded secret passed between male Aboriginal elders who presided over the harvest, artificial drying of the leaves and burning off of plants to increase yield. Claims by people familiar with the plants of this area that they do not produce fruit has sparked a hypothesis that the population contains only one or a few clones of ancestral individuals that have spread vegetatively and are largely, if not entirely, self-incompatible or sterile. If this population of *D. hopwoodii* were clonal, this would offer an explanation for the unique chemical properties of its leaves.

Abstract

The most highly sought after source of the native drug Pituri is a single population of *Duboisia hopwoodii* in south-western Queensland, near the Mulligan River. Inter-simple sequence repeats and sequence-related amplified polymorphisms were used to determine the genetic variation between 25 individuals from the Mulligan River population and a morphometric study investigated whether it is morphologically, as well as chemically, distinct. Results showed high genetic variability, refuting a hypothesis that the population contains only a few clones. However, two potential clones were identified from the genetic analysis, having only a small number of differences that could be explained by somatic mutations. The morphology of the Mulligan River population was found to be within the typical range for the variable *D. hopwoodii*.

***Keywords*:** clonality, nicotine, Pituri, Solanaceae, ISSRs, SRAPs

Muelleria 30(1): 65–71 (2012)

Figure 1. *Duboisia hopwoodii* plant from the study area (photo: N. Walsh)

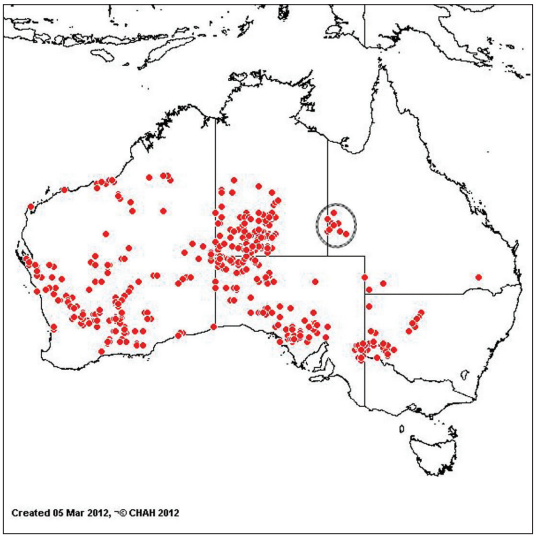

Figure 2. Distribution of *D. hopwoodii* with Mulligan River Region circled

Materials and methods

Genetic analysis

Leaf material was collected from 25 plants growing in Ethabuka Reserve, in the Mulligan River region, in the north-west of the Simpson Desert, in July 2010 (Figure 3a, b). Samples were taken along a transect about 20 km long (Figure 4). Samples were dried separately in paper bags placed in silica gel. DNA was isolated from 20-25mg of dried material using the QIAGEN plant DNeasy plant minikit. The manufacturer's protocol was followed except that leaf material was ground in a mortar and pestle with a small amount of sand.

As there is no genomic information available on *D. hopwoodii,* inter-simple sequence repeats (ISSRs) and sequence-related amplified polymorphisms (SRAPs) were used. These methods require no prior knowledge of the genome and have been successfully used on other members of the family Solanaceae (Li *et al.* 2010; Isshiki

Figure 3 a,b. Packing up plant presses, ready for the new day's collecting. (photo: N. Walsh)

et al. 2008; Kochieva *et al.* 2002). ISSRs have also been used for the identification of clones in many different taxa and for distinguishing between individuals in taxa where genetic variation may be limited (James and McDougall 2007; Li-Wang *et al.* 2008).

Nineteen ISSR and SRAP primer sequences (Li *et al*. 2010; Isshiki *et al*. 2008; Kochieva *et al*. 2002) were obtained from Invitrogen Australia and screened for consistent amplification of *D. hopwoodii* DNA fragments. Four primers were promising (UBC 888, FC8, M9 and M14; Table 1) and were used to amplify all samples.

Polymerase chain reactions (PCR) took place in 10µL reactions containing 5µL HotStarTaq Master Mix, 0.5 µL primer, 1µL DNA (20 – 40 ng) and 3.5µL H_2O. DNA amplification was performed in an Eppendorf MasterCycler gradient thermal cycler (Eppendorf, Hamburg, Germany). Annealing temperatures and ISSR cycles were based on the papers in which the primers were published or determined from a gradient PCR. PCR profiles for each primer are given in Table 2.

Table 1. Primers and sequences

Primer	Sequence 5'-3'
UBC 888	BDB(CA) 7
M9	(GACAC) 4
M14	(GACA) 4
FC8	GTGCTTTACTGTTTGCTCC

PCR products (10 µL) mixed with 2 µL of 6x loading dye were loaded into 2% agarose gel stained with ethidium bromide in 1x TBE (Tris-borate EDTA) buffer and run at 70 volts for between 2 and 4 hours depending on the size of the gel. DNA was visualised under UV light and scored

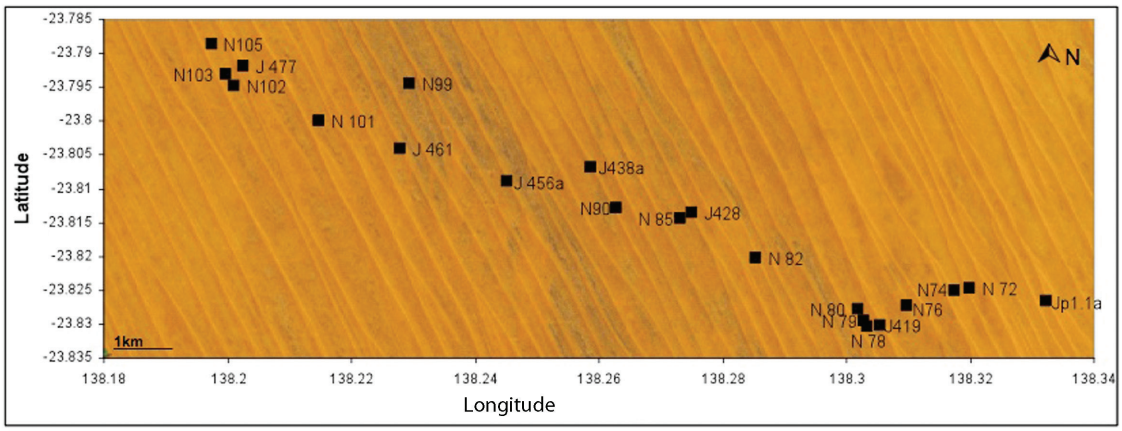

Figure 4. Satellite image of the study area showing orientation of sand dunes and the location of *D. hopwoodii* plants sampled for genetic analysis

Table 2. PCR protocols for each ISSR and SRAP primer used to amplify all samples *not used in final analysis

Primer	PCR protocols					
	Initial denaturation	Denaturation	Annealing	Elongation	PCR cycles	Final elongation
M14	15 min @ 95°C	30 sec @ 94°C	30 sec @ 42°C	30 sec @72°C	45	10 min @ 72°C
M9*	15 min @ 95°C	30 sec @ 94°C	30 sec @ 48°C	30 sec @72°C	45	10 min @ 72°C
UBC888	15 min @ 95°C	30 sec @ 94°C	30 sec @ 58°C	30 sec @72°C	45	10 min @ 72°C
FC8	15 min @ 95°C	30 sec @ 94°C	30 sec @ 35°C	30 sec @72°C	8	10 min @ 72°C
		+ 30 sec @ 94°C	30 sec @ 50°C	30 sec @72°C	32	

using Kodak Molecular Imaging Software (Eastman Kodak Company 2005). Each sample was repeated a minimum of two times to ensure repeatability of bands.

For each individual, the presence or absence of each DNA fragment was scored as "1" or "0", respectively. Bands that did not replicate consistently were not scored. Polymorphic bands from all useful primers were combined to generate one binary data matrix. One monomorphic band was removed from data before analysis. GenAlEx version 6.4 (Peakall and Smouse 2006) was used to perform a Mantel test, a Spatial Structure Analysis, a Matching Multilocus Analysis and an Allele Frequency Test. A Principal Co-ordinate Analysis (PCA; distance without data standardisation) was also performed from the genetic distance matrix and the geographic distance matrix using PATN version 3.03 (Belbin and Collins 2004).

Morphometrics

Herbarium specimens of *D. hopwoodii* from Western Australia, South Australia, the Northern Territory, New South Wales and Queensland were examined for morphological differences. Thirty-two specimens were measured for leaf length : width ratio, corolla length, inflorescence length, pedicel length, peduncle length, leaf shape and inflorescence branching pattern. The data generated were analysed with a multivariate analysis using NTSYSpc Version 2.11 (Rohlf 2000). Data for each character were range-standardised, such that the range of all characters equaled 1 unit, giving all characters equal weight (recommended by Milligan and Cooper 1988). Pairwise distances between specimens were calculated using the Manhattan metric, and the distance matrix subjected to clustering by the unweighted pair-group using arithmetic averages method (UPGMA; Sneath and Sokal 1973), and ordination using Non-metric Multidimensional Scaling (NMDS; Kruskal 1964a, b) in three dimensions. Specimens from Ethabuka reserve were also inspected for mature fruits.

Results

Genetics

Four primers (UBC 888, FC8, M9 and M14) amplified well with a high level of consistency across replicate pcr runs (Figure 5). M9 produced many bands too close together to score accurately so the locus was not included in further analysis. Each primer produced between 7 and 14 reliable bands giving a total of 33 bands, 32 of which were polymorphic (96.96%). The size of scored bands ranged from 330bp to 1300bp.

Twenty-five distinct genotypes could be recognised in the multilocus analysis. The lowest numbers of

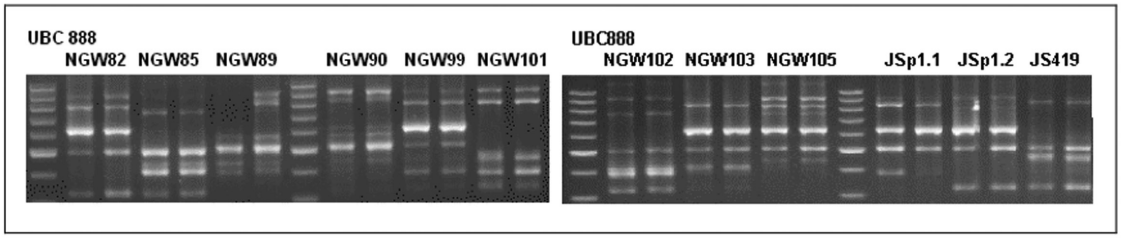

Figure 5. Replicates of samples 82 to 419 with primer UBC 888 showing consistency of bands and presence of polymorphisms. Unlabelled lanes contain Promega 100bp molecular size marker

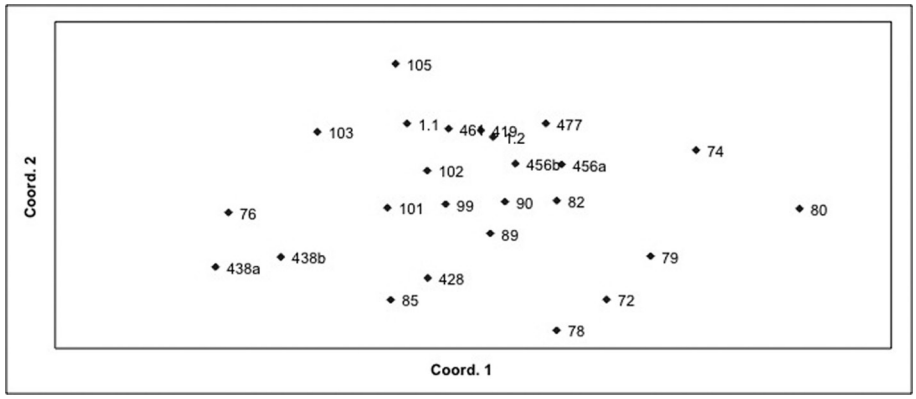

Figure 6. Principal Co-ordinate Analysis of genetic data via distance matrix without standardisation

different bands between two samples were 1 and 2 between two sets of adjacent plants; JS456a and JS456b were not more than 30 m apart whereas NGW85 and JS428 were located roughly 225 m apart. The maximum number of differences was 21 between NGW74 and JS438a, and NGW80 and JS438a. The PCA did not reveal any significant groupings of genotypes (Figure 6). The Spatial Structure Analysis did not find any correlation between geographic distance and genetic distance of samples, a finding which was supported by a Mantel Test (R^2=0.0007, P=0.381).

Morphometrics

The specimen from Ethabuka was not shown to be morphologically distinct from the other specimens examined, having a central position in the multidimensional ordination (Figure 7). In the UPGMA dendrogram the specimen from Ethabuka was grouped with other specimens from Queensland as well as specimens from New South Wales, the Northern Territory and South Australia. No fruiting specimens were found from the Mulligan River or Ethabuka regions.

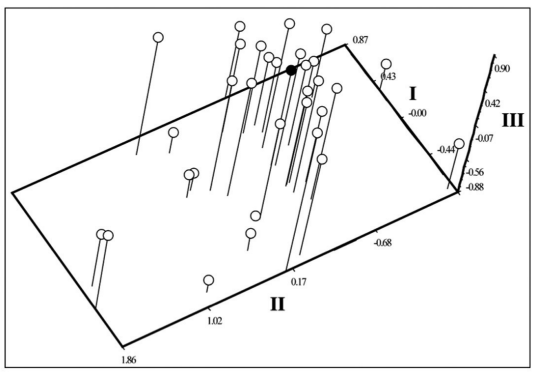

Figure 7. Spatial ordination of morphological characters. Specimen from Ethabuka Reserve black. Stress value = 0.097

Discussion

The brief morphological study undertaken did not find any morphological distinction between the population from Ethabuka and other populations across Australia that might suggest taxonomic distinctness to support the chemical uniqueness of this population. Nonetheless, considerable variation was seen within the species and it is possible that a more comprehensive study may detect morphological groupings across the entire species' range.

The genetic analysis has dispelled the hypothesis that the Ethabuka population contained only one or a few clones by finding 25 distinct genotypes from 25 samples. Therefore, the specimens sampled were established by seed and have since persisted vegetatively. However, JS456a and JS456b; and NGW85 and JS428, have only 1 and 2 differences respectively out of 32 bands, and could potentially be clone-mates with differences due to accumulated somatic mutations. As described by the genetic mosaicism hypothesis (Whitham and Slobodchikoff 1981), long-lived plants may accumulate somatic mutations in their individual "modules". The competition between modules of the same plant and consequential "natural pruning" of inferior modules are evolutionary pressures which result in a gradual change in genetic composition of the plant, creating a chimera. In this way, with somatic mutations as a source of

variation, perennial plants are able to evolve on a faster scale than the life span of a genet. The rate of somatic mutations has been predicted to be very low and unlikely to be detected (Gill *et al*. 1995) but Gross *et al*. (2012) clearly demonstrated somatic variation in ISSRs used to characterise clones of *Grevillea rhizomatosa* (Proteaceae). The likelihood of detection is predicted to increase with age of clones as well as the rate of somatic mutations. Examples of somatic mutation have been published for other plant species such as grape varieties that have been propagated clonally for more than 200 years (Crespan 2004; Hocquigny *et al*. 2004) and long-lived trees. Although the mutability of *D. hopwoodii* is unknown, the potato *Solanum tuberosum* L., also a member of the Solanaceae, has been shown to have high levels of somatic variability (Whitham and Slobodchikoff 1981). The age of the clones is also unknown but the distance between NGW85 and JS428 (225 m) suggests that they are quite old. These putative clones could be evidence for asexual reproduction occurring in the Ethabuka population and set the minimum potential for vegetative spread at 225 m. With more intensive sampling the maximum size of a clone could be determined.

Fire frequency is known to influence reproductive mode in plant species. Following destruction of the above-ground biomass of *G. rhizomatosa,* re-sprouting plants took 8 years to commence flowering (Gross *et al*. 2012). Pituri plants were burnt frequently by Aboriginal people to encourage new growth. It is possible that the burning regime has favoured plants that reproduce vegetatively over those that only reproduce via seed, resulting in the elimination of plants that rely solely on seed production. However, information is lacking on whether the extant clones are restricted to vegetative reproduction due to sterility.

The singularity of the Mulligan River population of *D. hopwoodii* could be investigated further through genetic analysis of typical, non-disjunct populations from Central and Western Australia. Although the Ethabuka population was found to be genetically diverse, a comparison with other populations could determine its relationship and an indication of its origin.

Acknowledgements

We would like to thank the Cybec Foundation for its generous support of PF and her project through the RBG Jim Willis Summer Studentship Program, Mike Bayly (University of Melbourne) and Niels Klazenga (Royal Botanic Gardens Melbourne) for their assistance with morphological data analysis, Mike Smith (National Museum of Australia) and Jen Silcock (University of Queensland) for valuable information regarding Pituri use and trade, and Andrew Harper (Australian Desert Expeditions) and other participants in the 2010 Ethabuka expedition, and the 20 wonderful camels that provided the means by which this expedition could be undertaken.

References

Barnard, C. (1952) The Duboisias of Australia. *Economic Botany* **6,** 3-17.

Belbin, L. and Collins, A. (2004). PATN version 3.02 and 3.03. Blatant Fabrications Pty Ltd., Hobart, Tasmania.

Crespan, M. (2004). Evidence on the evolution of polymorphism of microsatellite markers in varieties of *Vitis vinifera* L. *Theoretical and Applied Genetics* **108,** 231-237.

Gill, D.E., Chao, L., Perkins, S.L. and Wolf, J.B. (1995). Genetic Mosaicism in Plants and Clonal Animals. *Annual Rewiew of Ecology and Systematics* **26**, 432-444.

Gross, C.L., Nelson, P.A., Haddadchi, A. and Fatemi, M. (2012). Somatic mutations contribute to genotypic diversity in sterile and fertile populations of the threatened shrub, *Grevillea rhizomatosa* (Proteaceae). *Annals of Botany* **109,** 331-42.

Hocquigny, S., Pelsy, F., Dumas, V., Kindt, S., Heloir, M.C. and Merdinoglu, D. (2004). Diversification within grapevine cultivars goes through chimeric states. *Genome* **47**, 579-589.

Isshiki, S., Iwata, N. and Khan, M.M.R. (2008). ISSR variation in eggplant (*Solanum melongena* L.) and related *Solanum* species. *Scientia Horticulturae* **117**, 186-190.

James, E.A. and McDougall, K.L. (2007). Extent of clonality, genetic diversity and decline in the endangered mallee *Eucalyptus imlayensis*. *Australian Journal of Botany* **55**, 548-553.

Keogh, L. (2011). Duboisia Pituri: A Natural History *Historical Records of Australian Science* **22**, 119-214.

Kochieva, E.Z., Ryzhova, N.N., Khrapalova, I.A. and Pukhalskyi, V.A. (2002). Using RAPD for Estimating Genetic Polymorphism in and Phylogenetic Relationships among Species of the Genus Lycopersicon (Tourn.) Mill. *Russian Journal of Genetics* **38**, 958-966.

Kruskal, J.B. (1964)a. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* **29**, 1-27.

Kruskal, J.B. (1964)b. Nonmetric multidimensional scaling: a numerical method. *Psychometrika* **29**, 28–42.

Li, H., Chen, H., Zhuang, T. and Chen, J. (2010). Analysis of genetic variation in eggplant and related *Solanum* species using sequence-related amplified polymorphism markers. *Scientia Horticulturae* **125**, 19-24.

Li-Wang, L., Li-Ping, Z., Yi-Qin, G., Ming-Xia, W., Li-Ming, C., Jin-Lan, Y., Yan, W., Fan-Min, Y. and Long-Zhi, W. (2008). DNA fingerprinting and genetic diversity analysis of late-bolting radish cultivars with RAPD, ISSR and SRAP markers. *Scientia Horticulturae* **116**, 240-247.

Maiden, J H. (1893). The useful native plants of Australia. Trubner & Co: London.

Milligan, G.W. and Cooper, M.C. (1988). A study of standardisation of variables in cluster analyses. *Journal of Classification* **5**, 181-204.

Peakall, R. and Smouse, P.E. (2006) GenAlEx (version 6.4). Australian National University: Canberra.

Rohlf, FJ (2000). NTSYS-pc numerical taxonomy and multivariate analysis system (version 2.11T). Exeter Software: Setauket.

Roth, W.E. (1897). Ethnological studies among the north-west-central Queensland Aborigines. Government Printer: Brisbane.

Sneath, P.H.A. and Sokal, R.R. (1973). 'Numerical taxonomy: the principles and practice of numerical classification.' Freeman and Company: San Francisco.

Watson, P. (1983). This precious foliage: A study of the Aboriginal psychoactive drug Pituri. Sydney: *Oceania Monographs* Issue 26.

Whitham, T.G. and Slobodchikoff, C.N. (1981). Evolution by individuals, Plant-Herbivore Interactions, and Mosaics of Genetic Variability: The Adaptive Significance of Somatic Mutations in Plants. *Oecologia* **49**, 287-292.