DKCRC CP2.1 final report–Genetic diversity, trait variation and plant improvement

Michelle Waycott, School of Marine and Tropical Biology, James Cook University, Townsville QLD 4811.

Table of contents

Introduction

Fundamental to the development of novel food products with a distinctly Australian flavour is the identification and utilisation of the diversity that occurs in wild populations. In desert environments plants must survive extreme ecological stressors and it is not uncommon for the exposed portions of desert plants to be highly ephemeral and respond rapidly to environmental change. As a result desert plant life histories commonly show rapid responses to changing moisture availability, tolerance to extremes such as fire and prolonged drought, and biological mechanisms to persist until viable growth conditions occur, typically through seed banks, clonality or longevity. Thus, the standing stock (or population) of a particular plant in the field at any one time may be highly variable depending upon the history of local environmental conditions. As a result, the quantity of a particular plant, in the case of species such as *Solanum centrale*, known by a number of names, desert raisin, bush tomato or Akatyerr, will be highly variable for traits such as fruit production at any one time. The consequences to product supply will be high variability year to year reducing reliability of product availability at any one time. New product development and market acceptance requires reliable sources of marketable product preferably with highly valued palatability. At present, many desert bush foods are primarily obtained for market through wild-harvest activities, with some efforts to establish plantations and horticultural plots except for older products such as Macadamia. The wild harvest plant material collected is highly variable both in availability and palatability. Thus, for the benefit of industry development, we need to understand the basis of variability in plant characteristics, both desirable and undesirable. In addition, the use of wild collected material and subsequent

In this report, I summarise the results of investigations into the genetic resources, variability and factors affecting selections of plants for use in horticultural development. The final section of the report contains a set of recommendations and issues in the establishment of horticultural plantings of *Solanum centrale* for commercial production in Australia.

Chapter 1: Genetic diversity of natural stands of *Solanum centrale*

Fundamental to the development of new food products with a distinctly Australian flavour is the identification and utilisation identifiable variants that occur in the gene pool of a target species. There is typically a greater variability in any trait of interest, such as fruit size, taste, colour, among individuals in the wild than will ever be utilised in breeding programs. In desert environments plants must survive extreme ecological stressors and it is not uncommon for desert plants to be highly ephemeral and respond rapidly to environmental change. As a result desert plant life histories commonly exhibit a capacity to respond rapidly to changing moisture availability, tolerate extremes such as fire and prolonged drought, and to possess biological mechanisms to persist until viable growth conditions occur, typically through seed banks, clonality or longevity. As a result, the stock of a particular plant in the field at any one time may be highly variable depending upon the history of local environmental conditions. One consequence of this variability is that the quantity of plant product–in the case fruit of *Solanum centrale* and affiliates that are sold as desert raisin, bush tomato or Akatyerr–will result in variable fruit production at any one time and product supply is highly variable year to year. New product development and market acceptance requires reliable sources of marketable product preferably with highly valued palatability. At present, desert bush foods are primarily obtained for market through wild-harvest activities, with some efforts to establish plantations (Robins and Ryder 2008). To benefit industry development, improved understanding of the basis of variability in plant characteristics, both desirable and undesirable is essential.

Plant variability is determined by a combination of genetic and environmental factors. Many key plant traits are controlled by plant genotype. Traits such as palatability are the result of a combination of gene products, usually involved with plant anti-herbivory defences. One of the first steps in the production of cultivated lines is to establish the basis for plant variability in wild populations and identify a desirable plant "ideotype" or desired growth form. Taking this approach using morphological, environmental and genetic tools, a detailed understanding of how the plants vary may be established enabling selection of plant lines for putting into breeding programs and cultivation.

The research conducted aimed to contribute to the future success of both the wild harvest activity and the cultivation of bush tomato. Indigenous communities in the arid zone are interested in cultivation of these native food crops, and some have begun to do so. Given that the produce is, or can be, grown for both local community use and sale to the commercial sector, this research can potentially also benefit the industry as a whole in several ways.

Bush tomato exhibits characteristics suggesting considerable scope for improvement of planting material through selection and breeding. Prior to western cultures interest in native foods, plants such as Akatyerr had already been selected for over many centuries through aboriginal traditional land management practices. A recognition and contribution of both Aboriginal knowledge and scientific experimentation may be the most beneficial approach to both enhancing commercially desirable plant traits this species possesses and

preserving the natural resource present in ‘wild’ populations still harvested by Aboriginal peoples for food and for sale to commercial suppliers.

Solanum centrale is known to have a high degree of genetic and morphological variability based on field observations (Johnson, Ahmed *et al.* 2003) and through various attempts at horticultural plantings for fruit production over the past couple of decades. Attempts at horticultural plantings had been primarily focussed around planting seedlings for cropping within a 2 year period with limited success after this. To our knowledge seedlings were typically sourced from wild collected fruits and as a result plants grown were from variable locations and of variable quality. One of the first steps in the production of recognisable cultivated lines is to establish the basis for plant variability in wild populations and to identify a desirable plant, or ‘ideotype’. To do this, and to pave the way for the development of any Plant Varietal Rights (legally binding recognition of unique varieties), baseline information on plant trait variability are required.

Key traits that industry and researchers have identified as desirable include larger fruit size, fruit taste (sweeter), production of more synchronous crop and lower spininess. Ongoing market research will aid in the development of a more refined set of desirable traits however at present, baseline variability in any plant trait awaits testing for genetic versus environmental component. In addition, germination of *Solanum centrale* has been found to be generally low (Ahmed, JohnsoN *et al.* 2006; Johnson, Ahmed *et al.* 2003) and tissue culture techniques have been developed for *in vitro* generation of clonal material. However, the average land holder wishing to plant out *Solanum centrale* is unlikely to have access to such facilities. Practical tools for plant production systems are required for adequate application of new varieties that are established.

The first phase of research in this project has focused on developing genetic tools (markers) that can be used to document variation and also, later, to protect new IP (selected or improved plants). A methodology for measuring the bitter tasting toxin solanine which occurs in bush tomato fruit has been established (Maarten Ryder pers. comm. 2008; DKCRC Bush Produce project report project 2.1). This will aid in the selection of *Solanum centrale* for use in horticultural production which have a lower natural concentration of solanine. We have begun establishing natural variation in plant traits based on plants grown under identical conditions and will use material from these plants in future verification of the genetic markers identified by this study.

Two classes of genetic markers were used in the investigations into the genetic resources and plant improvement of *Solanum centrale*: DNA fingerprinting markers (ISSR’s–Inter Simple Sequence Repeats) and codominant markers (microsatellites). The different markers provide different insights into the genetic diversity and variation and are presented in separate sections below.

Part 1: DNA fingerprinting analysis

Introduction

The assessment of genetic diversity may be accomplished using markers that enable the evaluation of how similar or different individuals are within populations as well as providing insight into parentage and/or between populations relatedness and connectivity (Lowe, Harris *et al.* 2004). Analysis of a broad cross-section of an organisms genome is desirable when screening for genetic traits or relatedness (Lowe, Harris *et al.* 2004). Anonymous, arbitrarily amplified genetic markers are one class of markers that enables screening of a broad cross-section of genomes without significant startup in marker development and enabling analysis of many loci relatively easily (Bussell, Waycott *et al.* 2005). Inter-Simple-Sequence-Repeats (ISSRs) are one type of marker that utilises the almost ubiquitous presence of repetitive simple sequence DNA to anchor primers and enable anonymous, but reproducible amplification of diverse regions of the genome (Bornet and Branchard 2001; Wolfe, Xiang *et al.* 1998). High resolution analysis of ISSRs enables screening by using fluorescent tagged primers and running products on capillary DNA fragment analysers (Nagaraju, Kathirvel *et al.* 2002).

In this study we obtained material from *Solanum centrale* growing in the field, in garden plots and from commercial or semi-commercial fruit sources. We screened DNA obtained from these plant sources using a diversity of ISSR primer sets, resolving more than 90 loci through F-ISSR screening across more than 240 samples. The aim of this analysis was three-fold:

1. to evaluate the utility of ISSR markers for screening genetic diversity in Solanum centrale;
2. to identify the baseline genetic diversity able to be detected in *Solanum centrale* and relationships within and among populations;
   and
3. to determine spatial extent of *Solanum centrale* clones as evidence of reliance on vegetative growth.

Materials and Methods

Plant material

There was very little bush tomato growing in the wild in the 2004 – 2005 summer due to very dry conditions, the plant material available from field collected living plants was limited. As a result plant material was sourced from commercial batches of bush tomato fruit (Table 1). This material was not necessarily from a precise geographic location however, suppliers were able to provide general sources as outlined below.

Table 1: Locations from which samples were obtained for using in the genetics and plant improvement study. *=commercially sourced seed, ^=field collected plants.

Location		Latitude °S	Longitude °E	Number of samples analysed with ISSRs
Arrernte Kuyunba (same as Hatt Rd)	*	23.48	133.47	1
ASDP-Stirling	^	21.44	133.45	17
Central Anmatyerr (Outback)	*	22.8	133.16	7
Jamestown (same as Utopia)	*	22.14	134.46	3
John Holland	^	24.2	133.42	30
Hatt Rd	^	23.48	133.47	35
Murray Bridge (same as Outback = Central Anmatyerr)	*	22.8	133.16	5
Pine Hill	^	22.23	133.3	31
Tanami Rd	^	21.55	133.15	31
Western Anmatyerr (= Napperby)	^	22.46	132.47	60
Eastern Anmatyerr (= Utopia)	*	22.14	134.46	18
OzTukker	*	unknown		3

Genetic analysis

DNA was extracted from silica-dried leaf material using a SDS-Silica extraction method based on Epinstone et al. (2003) developed for this project to allow higher throughput screening.

Marker screening: the identification of highly polymorphic genetic markers for the purposes of DNA fingerprinting was based on interspersed simple sequence repeats (ISSRs). DNA is first extracted from fresh or dried leaves using Plant Tissue DNA Extraction Kit (Qiagen™, Epoch™). DNA is then quantified using spectrophotmetry, DNA amount standardised to 25 ng / µL and stored at -20°C until use. DNA fingerprints are generated using fluorescent tagged primers (Geneworks™) and PCR conditions to ensure consistency (detailed protocol Attachment C).

A total of 16 different primers were screened for amplification efficiency. A final set of six different amplification primers were used to determine individual plant genotypes. Six fluorescent tagged ISSR primers were used in single-primer PCR reactions: 818-hex, 826-hex, 889-tet, 855-tet, 888-fam, and Or-fam. Reagents used were Immolase Taq (Bioline, 5u/ml), 100x BSA (New England Biolabs) with 2 µl of DNA (20 ng) in a total 30 µl volume.

Primer sequences used in final screening (note letters correspond to international conventions for DNA sequences e.g. www.ncbi.nih.gov).

818 5’-CAC ACA CAC ACA CAC AG-3’
826 5’-ACA CAC ACA CAC ACA CC-3’
889 5’-DBD ACA CAC ACA CAC AC-3’
855 5’-ACA CAC ACA CAC ACA CYT-3’
888 5’-BDB CAC ACA CAC ACA CA-3’
OR 5’-GRTRCYGRTRCACACACACACACA-3’

Polymerase chain reaction using a MJ Research Peltier thermal cycler with an incubation step of 95°C for 10 minutes to activate the Immolase Taq followed by 15 cycles of 3 min at 94°C, 30 s at 94°C, 30 s at 64°C decreasing by 1°C per cycle. This was followed by 7 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. Final incubation was at 72°C for 5 min and the program was then held at 12°C indefinitely. PCR products were visualized on a 1.5% 1x TBE agarose gel. Products were quantified, multiplexed (889, 888 and 826 in one set, 818, 855 and Or in the second set) and cleaned using ethanol/ammonium acetate precipitations. Dehydrated products were sent to the Genetic Analysis Facility at James Cook University for genotyping.

Electropherograms were analysed using the Megabace Fragment Profiler v1.2 software. Peaks were scored for 97 loci (=unique fragment lengths for each primer and 1 = peak present; 0 = peak absent) for all samples. Genetic analysis was conducted using GenAlEx v6 software (Peakall and Smouse 2006).

Results and discussion

Genetic analysis of 241 plant samples across 97 loci revealed a high degree of genetic polymorphism (diversity) both within and between the populations studied evidenced by the number of bands (or loci) within populations (Figure 1). The level of genetic diversity detected was sufficient to verify genetic identity (i.e. individual fingerprints). Among the 241 samples, 217 genotypes were detected. Among the samples with shared multilocus genotypes, a high level of consistency was observed and we assign these as putative clones. Clones are only found among samples within a geographic location and nearly all clones detected were found in samples collected in the same location, on transects. The larger clones detected were at the John Holland and Hatt Road locations. At these sites up to 8 samples were observed to have shared genotypes. The samples collected were over larger spatial scales 10’s of metres and as such support field evidence that plants can achieve substantial vegetative colonisation using below ground structures.

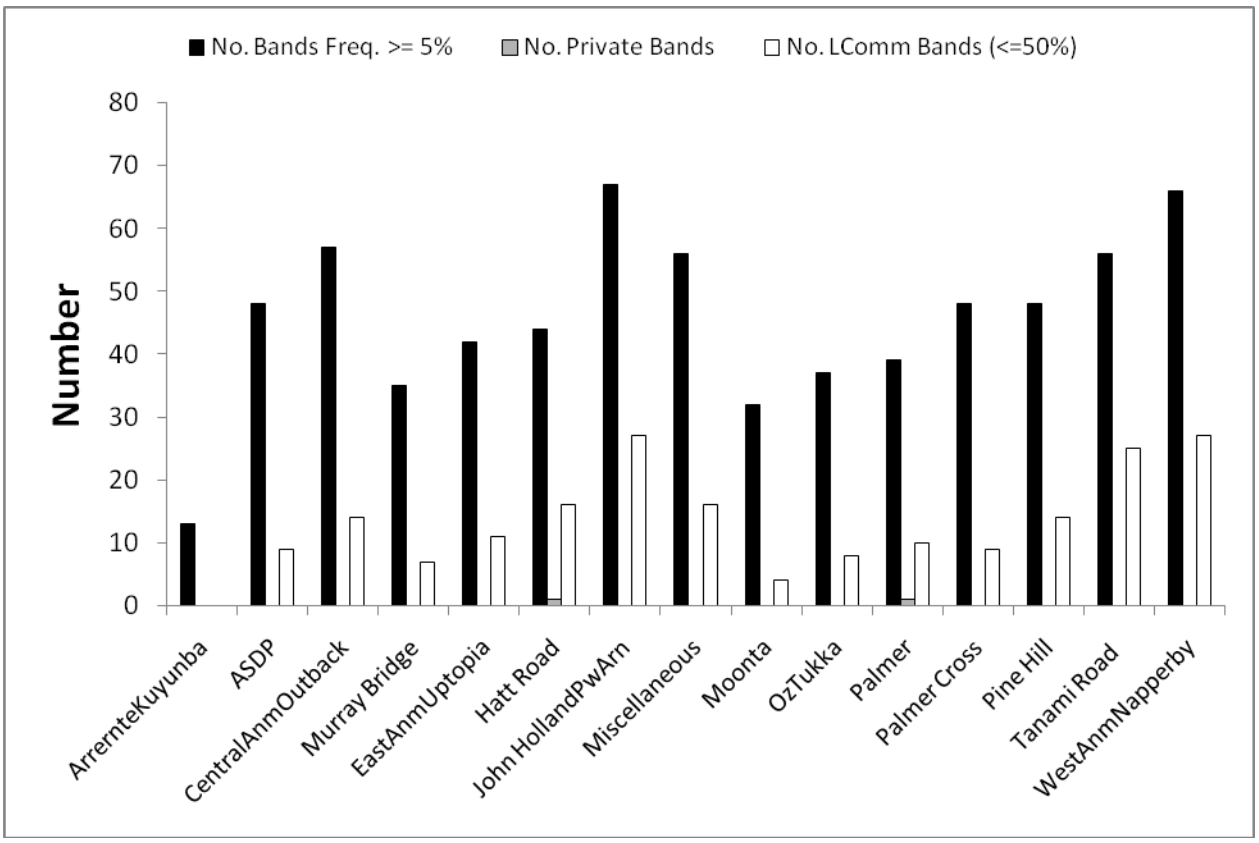

Figure 1: Average frequency of ISSR bands detected per populations and among seedlings from commercially sourced fruit across six primers and a total of 89 loci, locations refer to those listed in Table 1.

The genetic relatedness among the sets of samples collected suggests continuous variability and moderate degrees of connectivity (Figure 2). Some degree of population identity is emerging from the samples analysed. However, additional discrete population sampling is needed before a more robust interpretation of population identity can be made. If the observation of population structure is borne out across a wider population sampling, the likelihood of regionalised germplasm resources being identified is increased.

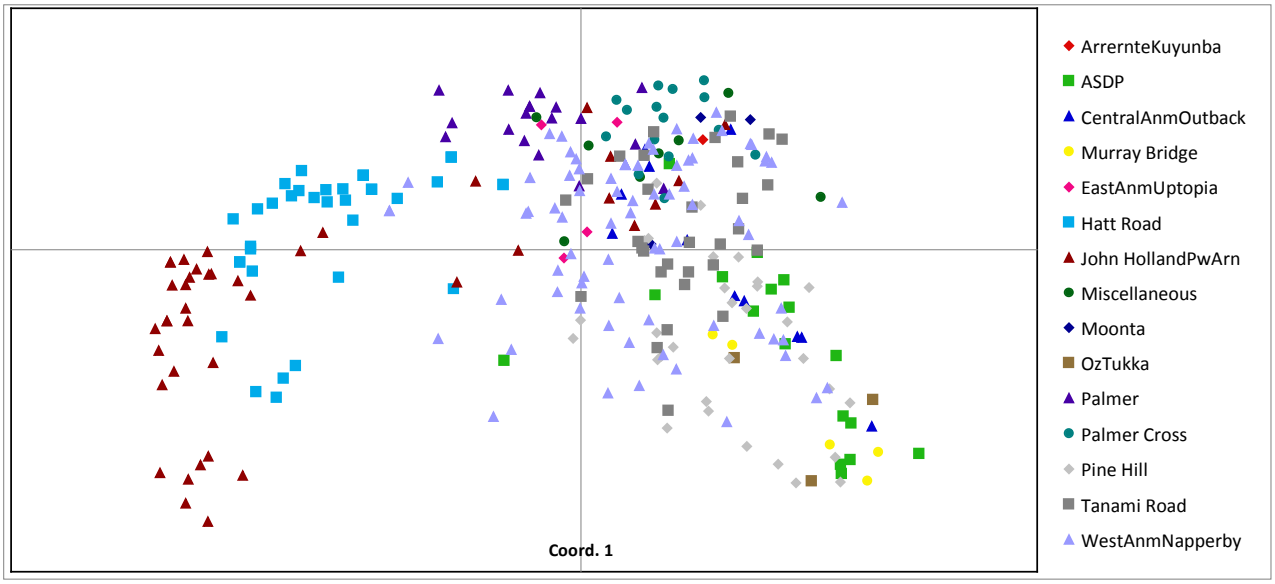

Figure 2: Principal coordinates analysis of genetic distance among all samples based on 97 ISSR loci across 15 different sets of samples (refer to Table 1 for sampling locations).*

The analysis is presented as two dimensional plots of a three dimensional analysis. The proportion of variation explained by each axis were 25.06% axis 1, 11.68% axis 2 and 10.19 axis 3.

The large proportion of variation is distributed among samples rather than among populations except for fruits obtained from plants in semi-cultivation. This is expected as cultivated plants are obtained from a more restricted line of genetic material.

The high level of variability with these markers suggests significant germplasm resources in the wild available for use in developing selected lines. Given the apparent capacity of this species to grow vegetatively forming extended clones in the wild, this growth strategy may provide useful mechanisms for use in horticultural development in selected lines. Future research may involve screening of potential horticultural lines for markers that identify them from among the bank identified here. This would aid in developing plant breeders rights and protecting unique varieties that are developed.

Part 2: Codominant marker (microsatellite) analysis

Introduction

Screening anonymous markers such as ISSRs as outlined in Part 1 of this chapter provides insight into relationships among sampled populations and also may be used to identify genetic markers or tags associated with specific genetic traits (Bussell, Waycott *et al.* 2005; Byrne 1996; Moran, Butcher *et al.* 2000). However, when assessing clonality, parentage and gene flow the use of codominant markers confers greater robustness and confidence to results obtained (Byrne 1996). At present, the codominant marker of choice for screening genetic variability are microsatellites (Garza and Wlliamson 2001; Queller, E *et al.* 1993; Ritland 2000;

Taylor, Horsup *et al.* 1997). The development of microsatellite markers involves screening broadly across the genome of interest, identifying regions containing simple sequence repeats (the 'microsatellite') and generating unique PCR primers to amplify the microsatellite region (Nunome, Suwabe *et al.* 2003a; Tautz 1989). A widely applied methodology in breeding programs, as well as in the screening of natural or wild populations this class of markers generates 'neutral' markers (i.e. they are not under direct selection) (Bataillon, David *et al.* 1996; Tautz 1989). Neutral markers allow evaluation of relatedness and population connectivity without assumptions as to patterns of inheritance (Ritland 2000). Typically, microsatellite markers are developed for the species to be analysed (i.e. they are species specific). However, significant success in using markers developed for closely related species has been made, particularly where a large number of comparable markers are available.

In the genus *Solanum*, a large number of commercially important agricultural species have been studied extensively. Tomato, potato, eggplant and tobacco are all well studied and numerous molecular markers identified and well characterised in these species (Jiang, Wang *et al.* 2009; Moon, Nifong *et al.* 2009; Nunome, Negoro *et al.* 2009; Nunome, Suwabe *et al.* 2003a; Nunome, Suwabe *et al.* 2003b; Rosa, de Campos *et al.* 2010; Ryzhova, Martirosyan *et al.* 2010). Screening of markers developed for confamilial species has yielded some success in identifying useful genetic markers (Selkoe and Toonen 2006). In addition, the development of species specific markers should yield high value data.

In this study we screened and identified genomic DNA libraries for highly polymorphic microsatellite markers specific to *Solanum centrale*. In addition, a suite of microsatellite markers developed for other species were evaluated for applicability in evaluating genetic diversity in *Solanum centrale*. The successful amplification of these loci allowed investigation of population genetic diversity, relatedness and phenotypic variability of central Australian *Solanum centrale*.

Materials and Methods

Plant material

The screening and development of codominant markers for *Solanum centrale* was conducted using material generated either as cuttings or seeds from planted material of the same plant material used in Part 1 (AZRI plants). Five population samples were also obtained from road verge populations around Alice Springs:

Site code	Latitude	Longitude
Pop 1	24°34'52.00"S	132°51'40.00"E
Pop 2	23°51'44.00"S	132°23'34.00"E
Pop 3	23°53'9.00"S	132°27'11.00"E
Pop 4	22° 7'48.00"S	133°25'18.00"E
Pop 5	21°12'56.00"S	134° 8'20.00"E

In addition, plants were purpose grown in shade house conditions for testing genotype by environment interactions between soil nutrients and seedlings from the same maternal source were screened sourced from plants grown at the AZRI plantation.

DNA Extraction

Total genomic DNA was extracted from dried leaf tissue using Gencatch gel extraction kits (Epoch Biolabs) according to a modified version of the manufacturers instructions. Initial grinding step was performed on dried leaf material in 2ml eppendorf snap-lock tubes with 1mm zirconia/silica beads in place of mortar and pestle grinding under liquid nitrogen. Instructions were adhered to up to applying samples to individual silica membrane spin columns. At this step these were replaced with 96-well silica membrane plates. Subsequent wash steps were performed in these plates and DNA was eluted into a 96-well PCR plate.

PCR amplification of microsatellite loci

A genomic DNA library enriched for multiple simple sequence repeats was constructed following a modified version of Glenn and Schable (Glenn and Schable 2005), in which the Hamilton SNX linker system was utilized (Hamilton, Pincus *et al.* 1999). Nuclear genomic DNA was obtained from multiple individuals for library construction. Amplified SNX linker-ligated DNA fragments were ligated into plasmid DNA (pMOSBlue, GE Healthcare), then transformed into pMOSBlue competent cells (GE Healthcare) and plasmid inserts were PCR amplified with T7 and U19 vector primers. PCR products were visualized and fragment length estimated using gel electrophoresis. Clones which contained PCR products greater then 300 bp long were gel purified (Epoch Biolabs, Sugar Land, TX) and sequenced using DYEnamic™ ET Dye Terminator sequencing chemistry (GE Healthcare) and analyzed on a MegaBACE 1000 system (GE Healthcare). Few of the sequences obtained from clones yielded uninterrupted microsatellites, and of those only 7 were suitable for primer design. Primer design was performed in Primer 3 (Rozen and Skaletsky 2000) and in addition to the design of novel primers, 48 pairs of existing primers for microsatellite loci in other *Solanum* species were selected from the literature (Benor, Zhang *et al.* 2008; Frary, Xu *et al.* 2005; Nunome, Suwabe *et al.* 2003a; Nunome, Suwabe *et al.* 2003b; Stagel, Portis *et al.* 2008). Primer selection criteria included product size expected to be between 100 and 400 bp as well as possessing simple uninterrupted repeats, not complex or compound repeats. Where data was available weighting was given to markers shown to cross amplify in additional species to the one it was designed, and markers that showed higher numbers of alleles in their given species. Primers were obtained from commercial providers (GeneWorks™, Adelaide, SA and Invitrogen™, Carlsbad, CA, Table 2) with the forward primer of each pair synthesized with an M13 tail (5'-CACCACGTTGTAAAACGAC-3') to facilitate easy automated screening (Boutin-Ganache, Raposo *et al.* 2001). Primers were tested under a range of amplification conditions and visualized by gel electrophoresis to determine which produced a single product of appropriate length in *S. centrale* (Table 2).

TABLE 2. Characteristics of 33 microsatellite loci shown to amplify a single product in *Solanum centrale*. Shown for each primer pair are the forward and reverse sequence, repeat type, original size fragment (bp) and annealing temperature (T_a).

Marker	Sequence	Repeat	Size (bp)	T_a (ºC)
[a]SSR135	F:*TGATCGCTTGTGTCCACCTA	$(ATT)_6$	133	56
	R:AAAGGAAGTGATGGAAAGCG			
[a]SSR150	F:*ATGCCTCGCTACCTCCTCTT	$(CTT)_7$	217	64
	R: AATCGTTCGTTCACAAACCC			
[a]SSR96	F:*GGGTTATCAATGATGCAATGG	$(AT)_{12}$	222	59
	R:CCTTTATGTCAGCCGGTGTT			
[a]SSR26	F:*CGCCTATCGATACCACCACT	$(CGG)_6$	179	57
	R:ATTGATCCGTTTGGTTCTGC			
[a]SSR32	F:*TGGAAAGAAGCAGTAGCATTG	$(TTC)_7$	187	56
	R:CAACGAACATCCTCCGTTCT			
[a]SSR14	F:*TCTGCATCTGGTGAAGCAAG	$(ATA)_9$	166	63
	R:CTGGATTGCCTGGTTGATTT			
[a]SSR11	F:*CCTTCAATTGACCTCCCTCA	$(CAG)_6$	235	61
	R:GCATCTGGAAATTAGAGGCG			
[a]SSR593	F:*TGGCATGAACAACAACCAAT	$(TAC)_7$	295	56
	R:AGGAAGTTGCATTAGGCCAT			
[a]SSR47	F:*TCCTCAAGAAATGAAGCTCTGA	$(AT)_{14}$	191	56
	R:CCTTGGAGATAACAACCACAA			
[a]SSR350	F:*GGAATAACCTCTAACTGCGGG	$(AT)_{13}$	267	58
	R:CGATGCCTTCATTTGGACTT			
[a]SSR594	F:*TTCGTTGAAGAAGATGATGGTC	$(TCT)_8$	293	55
	R:CAAAGAGAACAAGCATCCAAGA			
[a]SSR4	F:*TTCTTCGGAGACGAAGGGTA	$(CGG)_7$	168	60
	R:CCTTCAATCCTCCAGATCCA			
[a]SSR218	F:*GTGGTTATCCCAAGACCCAA	$(TCA)_7$	127	60
	R:CGCCAGTCTTCCTCTGACTT			
[a]SSR248	F:*GCATTCGCTGTAGCTCGTTT	$(TA)_{21}$	251	61
	R:GGGAGCTTCATCATAGTAACG			
[a]SSR20	F:*GAGGACGACAACAACAACGA	$(GAA)_8$	157	61
	R:GACATGCCACTTAGATCCACAA			
[b]EM114	F:*AGCCTAAACTTGGTTGGTTTTTGC	$(AC)_{13}$	221	65
	R:GAAGCTTTAAGAGCCTTCTATGCAG			
[b]EM155	F:*CAAAAGATAAAAAGCTGCCGGATG	$(CT)_{38}$	248	65
	R:CATGCGTGAGTTTTGGAGAGAGAG			
[c]EEMS21	F:*TGATGTTGAACCGACACAAGA	$(AGA)_5$	131	56
	R:CGTCTTCATCTTCCTCCTCG			
[c]EEMS22	F:*GAAGGACGTTGGTCCTGGTA	$(AAG)_5$	162	60
	R:CTGTTCATTATCCCCATCGC			
[c]EEMS24	F:*CACCTGTTTGAGCACCTTGA	$(CTT)_5$	221	60
	R:CACCGAAGGCAGAGAAGAAG			
[c]EEMS25	F:*CCCATAGCTTTGCTCGAGAT	$(CTT)_5$	227	61
	R:GCACCAAAGGCAGAGAAGAA			
[c]EEMS26	F:*GACACTCCCCTACTTCCACCT	$(CTT)_5$	260	59
	R:CGCTTAGCAGAAGCCGATAA			
[c]EEMS29	F:*TCAGTCAACTGCATCACCAGA	$(ATG)_5$	118	60
	R:ATTCCCATTATTGGCTGCTG			
[c]EEMS31	F:*GAGAAGTTGGCTTCAGTGCC	$(TGG)_5$	239	65
	R:TAAACTCAAGGGATGCTGGG			
[c]EEMS38	F:*TTCAATCGAACTTCGGAACC	$(CAC)_5$	148	62
	R:ATGACGGTGGATCTCGCTAC			
[c]EEMS39	F:*GGAGAGATGGATGCCGAATA	$(CTG)_5$	166	62
	R:TCTCGACCTTAGCCTGCATT			
[c]EEMS42	F:*GCTCAGCAACCACAGTACCA	$(GCA)_6$	152	65
	R:GTCCGGACTTCATCAGCATT			
[c]EEMS44	F:*CCTTCAAACCCTCTCCCTTC	$(GCC)_5$	216	64
	R:GTGAAACGTGGTGGAGGTCT			
[d]AI773078	F:*GATGGACACCCTTCAATTTATGGT	$(AAT)_{14}$	145–190	62
	R:TCCAAGTATCAGGCACACCAGC			
HQ203033	F:*GACAGAGGCTTGTTCTGCTTG	$(CAA)_4$	214	55
	R:AGGGTTCCCAGGAAACAGTC			

HQ203034	F:*GTTCTCCCTCTGATTGTTC R:GTAGCATGGTTTCACTCTTG	$(ATC)_{20}$	246	55
HQ203035	F:*CAATTTCAAAACAAAAGAAC R:GTCCAATGCCTCTAGTGT	$(TCA)_5$	238	56
HQ203036	F:*TTGCTAGCAGGTTGGGACTT R:CAAAAGCAAATCCCGCTTAG	$(TTC)_{24}$*	252	60

NOTES: *Forward primer synthesized with an M13 tail of 5'-CACCACGTTGTAAAACGAC-3'
[a] First described in Frary et al. (2005), [b] first described in Nunome et al. (2003), [c] first described in Stagel et al. (2008), [d] first described in Benor et al. (2008).

Single band products were screened against 12 *S. centrale* individuals and analyzed on a MegaBACE 1000 system (GE Healthcare). Markers that produced unscorable, single fixed allele or fixed heterozygote genotypes were discarded and the the remaining markers were used to screen five popultions sampled from central Northern Territory, Australia. PCR conditions for 20 µl reactions using the primers HQ203036 and SSR11 included 1X reaction buffer, 50 ρmol $MgCl_2$, 2.5 ρmol of dNTPs, 1.0 ρmol of BSA IV, 1.25 ρmol of M13 labeled forward primer, 5 ρmol of the reverse primer, 5 ρmol of fluoro-labeled M13 primer and 0.5 units of hot start *Taq* DNA polymerase. EEMS21 and SSR32 had 1X reaction buffer, 30 ρmol $MgCl_2$, 2.5 ρmol of dNTPs, 1.6 ρmol of BSA IV, 1.5 ρmol of M13 labeled forward primer, 6 ρmol of the reverse primer, 6 ρmol of fluoro-labeled M13 primer and 0.5 units of hot start *Taq* DNA polymerase. HQ203035 varied from this containing 50 ρmol of $MgCl_2$ and EEMS42 had standard *Taq* DNA polymerase. Thermocycler conditions were 95°C for 10 min followed by 35 cycles of 30 s at 95°C, 30 s at specific annealing temperature, then 30 s extension at 72°C, followed by final 10 min extension at 72°C. HQ203035, EEMS21 and SSR32 were performed with an annealing temperature of 56°C, HQ203036 at 60°C, SSR11 at 61°C and EEMS42 had 3 min at 95°C instead of 10 min to begin with and a annealing temperature of 65°C. A summary of amplification conditions is listed in Table 3.

Table 3. Microsatellite locus PCR amplification conditions for loci included in final screening, primer code sources included in Appendix B.

Locus (full primer list attached in appendix B)	H_20 (µL)	10x PCR buffer (µL)	$MgCl_2$ (µL)	dNTPs (µL)	BSA (µL) 1 mg/mL	F primer (10µM) with tail volume µL	R primer (10µM) volume µL	Universal labelled primer (10µM) volume µL	Enzyme (5U/µL, [I]=immolase, [B]=biotaq) volume µL
Solcen02	14.275	2.00	1.00	0.25	0.25	0.125	0.50	0.50	0.10 [I]
Solcen06	14.425	2.00	1.00	0.25	0.10	0.125	0.50	0.50	0.10 [I]
Sol03	14.425	2.00	1.00	0.25	0.10	0.125	0.50	0.50	0.10 [I]
Sol10	14.425	2.00	1.00	0.25	0.10	0.125	0.50	0.50	0.10 [I]
Sol18	14.140	2.00	1.00	0.25	0.16	0.150	0.60	0.60	0.10 [B]
Sol45	14.140	2.00	1.00	0.25	0.16	0.150	0.60	0.60	0.10 [B]
Solcen05	14.140	2.00	1.00	0.25	0.16	0.150	0.60	0.60	0.10 [I]
Sol32	14.540	2.00	0.60	0.25	0.16	0.150	0.60	0.60	0.10 [I]
Sol08	14.540	2.00	0.60	0.25	0.16	0.150	0.60	0.60	0.10 [I]
Sol46		2.00	0.60	0.25	0.16	0.150	0.60	0.60	0.10 [B]
Sol18	14.247	2.00	1.00	0.25	0.16	0.043	0.60	0.60	0.10 [B]

Results and Discussion

A total of 66 alleles were detected across all loci for samples screened across five natural, road verge, populations of *Solanum centrale* from central Australia. A total of 80 alleles were detected among the seedlings grown from a single maternal plant from the AZRI plot in Alice Springs (Table 4). Due to the identification of polyploidy among the samples screened, it was not possible to calculate the commonly used measures of genetic variability. Instead, GenoDive (Meirmans and Van Tienderen, 2004) was used to apply statistics appropriate for comparisons among polyploidy populations, the gene diversity statistic (Nei, 1987) for the individual populations and the corrected total heterozygosity measure (Nei, 1987) to asses the expected frequency of heterozygotes across all populations. Both of these measures are sample estimates and permutations were set to 9999. Gene diversity ranged from 0.11 to 1.66 and total expected heterozygosity from 0.59 to 0.89 (Table 5).

Table 4. Allelic diversity detected in loci screened across two samples sets of *Solanum centrale* from natural populations in central Australia and from large fruits obtained from a single maternal plant in the AZRI plot in Alice Springs.

Locus	number alleles detected in natural populations screened	number alleles detected among AZRI seedlings from large fruited maternal plant
S08	7	21
S32	8	7
SC05	12	16
S45	13	12
SC06	12	24
S10	14	NA
Total	66	80

Table 5. Levels of polymorphism and gene diversity for highly polymorphic loci across five populations of *S. centrale*. Number of alleles (A), gene diversity (H_S), the overall number of alleles per each locus (A_t), the range of fragment lengths and corrected total heterozygosity (H_T).

	Jay (40) 24°34'52"S 132°51'40"E		Ell (42) 23°51'44"S 132°23'34"E		Em (40) 23°53'9"S 132°27'11"E		En (29) 22°7'48"S 133°25'18"E		Ohh (41) 21°12'56"S 134°8'20"E		Overall (192)		
Marker	A	H_S	A	H_S	A	H_S	A	H_S	A	H_S	A_t	Size (bp)	H_T
SSR32	3	0.11	7	0.71	5	0.69	3	0.52	3	0.39	7	177–198	0.59
EEMS21	4	0.63	5	1.66	5	0.92	4	0.55	5	1.25	8	130–160	0.66
HQ203035	5	0.26	5	1.39	7	1.02	7	0.76	6	0.71	12	230–275	0.68
EEMS42	12	1.09	7	1.02	6	0.99	6	0.76	8	0.99	13	175–208	0.85
HQ203036	6	1.01	4	0.71	7	1.58	9	0.86	10	1.00	12	251–293	0.83
SSR11	10	0.88	10	0.84	8	0.84	7	0.81	7	0.79	14	230–287	0.89

The most significant result, other than the detection of a high level of genetic diversity among the samples screened was the observation than nearly all samples analysed were determined to be polyploid. Polyploidy is common among flowering plants (Otto and Whitton 2000), however, mixed ploidy levels within species can lead to genetic disruption in populations (Young and Murray 2000). The data generated here provides evidence of at least two ploidy levels present among the samples being utilised for horticultural development (Appendix C). Polyploid analysis using microsatellite markers has been demonstrated in potato (Sharma, Sarkar *et al.* 2010).

Due to the polyploid nature of the data, many standard analyses are not possible. Among the samples obtained from wild populations using a Bruvo distance genetic analysis implemented in Genodive (Bruvo, Michiels *et al.* 2004), a Principal Coordinates Analysis was conducted to assess the overall relationships among samples as assigned to the populations collected from (Figure 3). A similar pattern was detected to that found with ISSR (DNA fingerprinting) markers where some populations were readily separated as different genetically (populations 4 and 5) were identified and others form a continuum of genetic distinctiveness (populations 1-3). Interestingly these correspond to populations South of the McDonnell Ranges (populations 1-3) versus North of the ranges (populations 4 and 5).

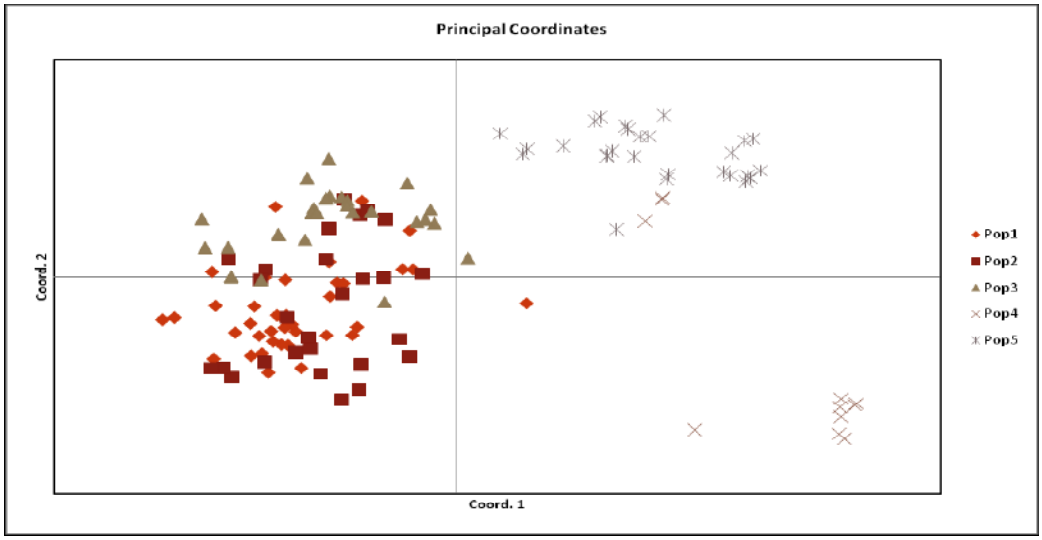

Figure 3. Two dimensional plot of the Principal Coordinates Analysis of microsatellite marker generated genotypes among five samples locations across central Australia. Populations were obtained from locations listed above.

Total genetic diversity among plants grown at the AZRI plot and five populations of *Solanum centrale* near Alice Springs was high. This high diversity was complicated by the finding that samples were also found to be polyploid. The genetic signature of the polyploidy identified was that of at least tetraploid (4x) or hexaploid (6x). Although some effort was made to confirm ploidy levels using cytological methods, material for observing chromosomes (flower buds) was not available. The confirmation of ploidy levels is a matter of considerable importance as it will influence plans for horticultural development. Mixed ploidy had already been observed in arid zone *Solanum* species (Randell and Symon 1976) and an evaluation of the genetic

diversity of populations with respect to ploidy levels will be essential to selection of materials for using in any breeding program. In addition, species concepts should take ploidy levels into consideration.

Part 3: Preliminary phylogenetic analysis of arid zone Australian *Solanum* species

A preliminary phylogenetic analysis was conducted to confirm the relationships among arid zone *Solanum* species to confirm general species concepts in the group being utilised as potential materials in the bush foods industry. A DNA barcoding approach was utilised following protocols established by the Royal Botanical Gardens Kew (http://www.kew.org/barcoding/). DNA sequencing followed the protocols set out by the Kew Barcoding initiative. Specimens were collected from cultivated materials in the Alice Springs Desert Park, from herbarium specimens and from field collections.

Phylogenetic analysis to confirm species resolution was conducted using multiple loci (Table 6) to determine relationships among a subset of samples collected for genetic analysis and other closely related species in central Australia. DNA sequences generated using standard protocols for the Megabace 1000 (at the James Cook University genetic analysis facility) were analysed using minimum evolution and parsimony methods implemented in MEGA v4 with 1000 bootstrap replicates to determine support for trees reconstructed from the five loci screened in a final combined analysis.

Table 6. Primers used to amplify loci used in phylogenetic analysis of *Solanum* species from central Australia: all loci are from the chloroplast genome–rpoB, rpoC, accD and YCF5 pimers were those described by the Plant DNA barcoding initiative (Royal Botanical Gardens Kew), trnL primers those described by (Taberlet *et al.* 1991).

Gene	Primer	Direction	Sequence 5'-3'
rpoC	1	f	GTGGATACACTTCTTGATAATGG
	4	r	CCATAAGCATATCTTGAGTTGG
rpoB	1	f	AAGTGCATTGTTGGAACTGG
	4	r	GATCCCAGCATCACAATTCC
accD	1	f	AGTATGGGATCCGTAGTAGG
	3	r	TTTAAAGGATTACGTGGTAC
YCF5	1	f	GGATTATTAGTCACTCGTTGG
	4	r	CCCAATACCATCATACTTAC
trnL	c	f	CGAAATCGGTAGACGCTACG
	f	r	ATTTGAACTGGTGACACGAG

Phylogenetic analysis revealed that the current taxonomic entity known as *Solanum centrale* is comprised of more than one taxon (Figure 4). This in combination with the distinctiveness of some populations samples suggests a complexity in the genetic structure of populations of this species. It also creates several complex issues for the horticultural development of this species which will be discussed at the end of this report. This preliminary phylogenetic analysis provides additional evidence of the complex genetic structure of the species *Solanum centrale*. Further evaluation of phylogenetic relationships among arid zone *Solanum* species will be useful in improving our understanding of the systematic relationships across this group.

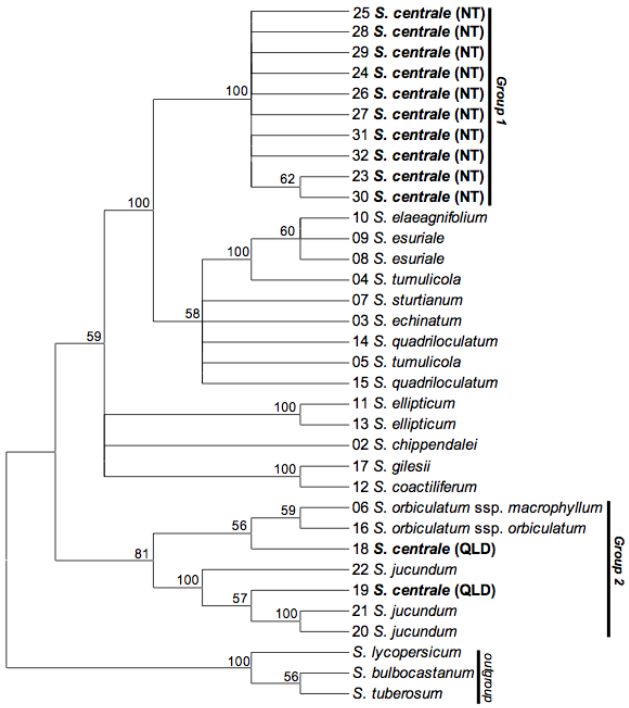

Figure 4. Minimum evolution 50% consensus cladogram based on 1000 bootstrap replicates, depicting hypothetical relationships based on DNA sequence data for five chloroplast loci in central Australia *Solanum* species.

Chapter 2: Morphometric variation of shadehouse grown *Solanum centrale*

Introduction

The documentation of morphological variation within a species destined for horticultural development is important in establishing baselines of natural variability for selection programs to operate on. Phenotypic plasticity is a common mechanism plants adopt when faced with changing environments (Agrawal 2001; Bradshaw 1965; Scheiner 1993). Desert plants must cope with extremes of environmental fluctuations, temperature and moisture availability in particular (Albrecht, Escobar *et al.* 2010; Freas and Kemp 1983; Kadmon 1993). Documenting the scale of phenotypic responses to changing environmental conditions is a first step towards identifying environmental versus genetic sources of plant traits of interest. In this study baselines of morphological plasticity during the early growth phase of *Solanum centrale* plants was conducted using shadehouse growth trials.

Materials and methods

Experiment 1: Seedlings raised during germination trials were grown to flowering under standard shadehouse conditions at James Cook University in Townsville, Qld. Plants were sourced from fruited obtained in Tanami Road, Kuyanba Road, Pine Hill, Alice Springs Desert Park, *S. cleistogamum* (ASDP), Outback, Oztukka, and Utopia. The plants were grown in 2.6 L pots containing 4 coarse sand: 1 loam: 1 vermiculite: 1 pearlite. The pots were fertilised with dilute Miracle Grow (1.25 mL in 2 L) monthly and treated with fungarid fortnightly while plants were small and then monthly. Morphological characters of plants were measured over a period of 1 week and measurements were taken before temperatures were stressful to plants in the morning. Characters measured were leaf angle (° to vertical), leaf area (mean of 3 replicates on the 4th leaf and below from the main growing shoot), leaf length (on the 4th leaf from the apex of the main growing shoot), leaf breadth (as for leaf length), number of spines in 1 cm span below (on the 4th leaf from the apex of the main growing shoot), spine length (the longest spine in the area scored for spine number), reflectance (using an integrated sphere), % reflectance.

Experiment 2: We examined the effects of different nutrient regimes on morphology and growth characteristics during the early developmental stages as measured by total plant growth. A 3 level nutrient regime, with water levels remaining constant where nutrient treatments included no added nutrients, standard commercial nutrient loading and double loading (T. Collins pers. comm.). A random selection of *Solanum centrale* fruits were collected from plants grown at the Arid Zone Research Institute in the DKCRC Bush produce field trial. Plants located at the AZRI horticultural plot originated from a number of geographic locations, Napperby, Ambilindum and Utopia. Each fruit selected for use in the growth trial was selected to fit into 1 of 4 fruit size classes. The classes were defined by weight, ranging from 0.350-0.450g, 0.700-0.800g, and 0.945-1.391g. The fourth class consisted of seeds from a single large fruited maternal plant

where fruits were consistently larger and heavier, with all fruit ranging from 1.327-1.465g. For each nutrient treatment fruits collected from the AZRI growth trial were grown, at least 9 replicates per fruit for each of the three treatments, a total of 27 seeds per fruit being grown. Fruit of different were grown, a total of 540 plants across the three treatments.

Fruit were soaked in water for 1-4 hours, with time dependent on fruit size, larger fruit requiring longer soaking before seed extraction. Seeds were extracted from fruit pulp in a shallow tray of water, and then air dried on paper towel at 28ºC overnight. Seed number and fruit weight were recorded. Seeds were soaked in a solution of 500mg Gibberellic acid (GA) per 1L of distilled water for a period of 24 hours to enhance germination rates. After GA treatment, seeds were planted out into seedling trays measuring, with seeds from each fruit in an individual, labeled tray. After germination, individual seedlings were planted out into labeled 140mm pots containing 1 of 3 nutrient levels within a standardized potting mix (control/zero nutrients—0.6g/L of wetting agent per litre soil, standard nutrients—4.5g/L osmocote exact standard, 0.463g/L FeSO4, 0.6g/L wetting agent, 0.7g/L osmoform premix, double nutrients—2x dose all components. The pots were arranged in a randomized block design within a greenhouse located at the Alice Springs Desert Park three posts wide on each of the two benches. Wire cages were constructed to prevent grasshopper attack. Date of germination was recorded for each plant to enable the collection of data relating to time based measures of performance. Total plant above ground biomass was recorded as a dry weight at the end of 7 months of growth (note plants grow very slowly during winter months) at the Alice Springs Desert Park.

Experiment 3: Fruits from the single large fruited maternal plant identified at the AZRI plot were grown in glasshouse conditions under similar conditions to Experiment 2. In addition to plant biomass morphometric measurements of plants were taken during the growth period.

Results and discussion

Experiment 1: Assessment of morphological variability revealed high levels of plasticity among the 237 plants surveyed from 35 fruits from fruit sourced in 8 locations (Figure 2.1). Sample Sc17 was germinated from fruit collected off *Solanum cleistogamum*, this sample was obviously different for example was more spiny and smaller sized leaves. Among all traits measured the variability was a great within fruits as between them. Some between fruit differences were significant for leaf reflectance. This is a measure of how much light is reflected from the surface of leaves and corresponds with leaf pubescence (hairiness) and the reflective quality of the hairs and leaf surface (colour). These characteristics correspond with leaves which had copper coloured hairs versus silver hairs. Highly variable levels of morphological variation within fruits from the same plant were detected for all measures screened. Future studies of variability should concentrate on production relevant characteristics such as total plant biomass, fruit weight, fruit size or fruit taste.

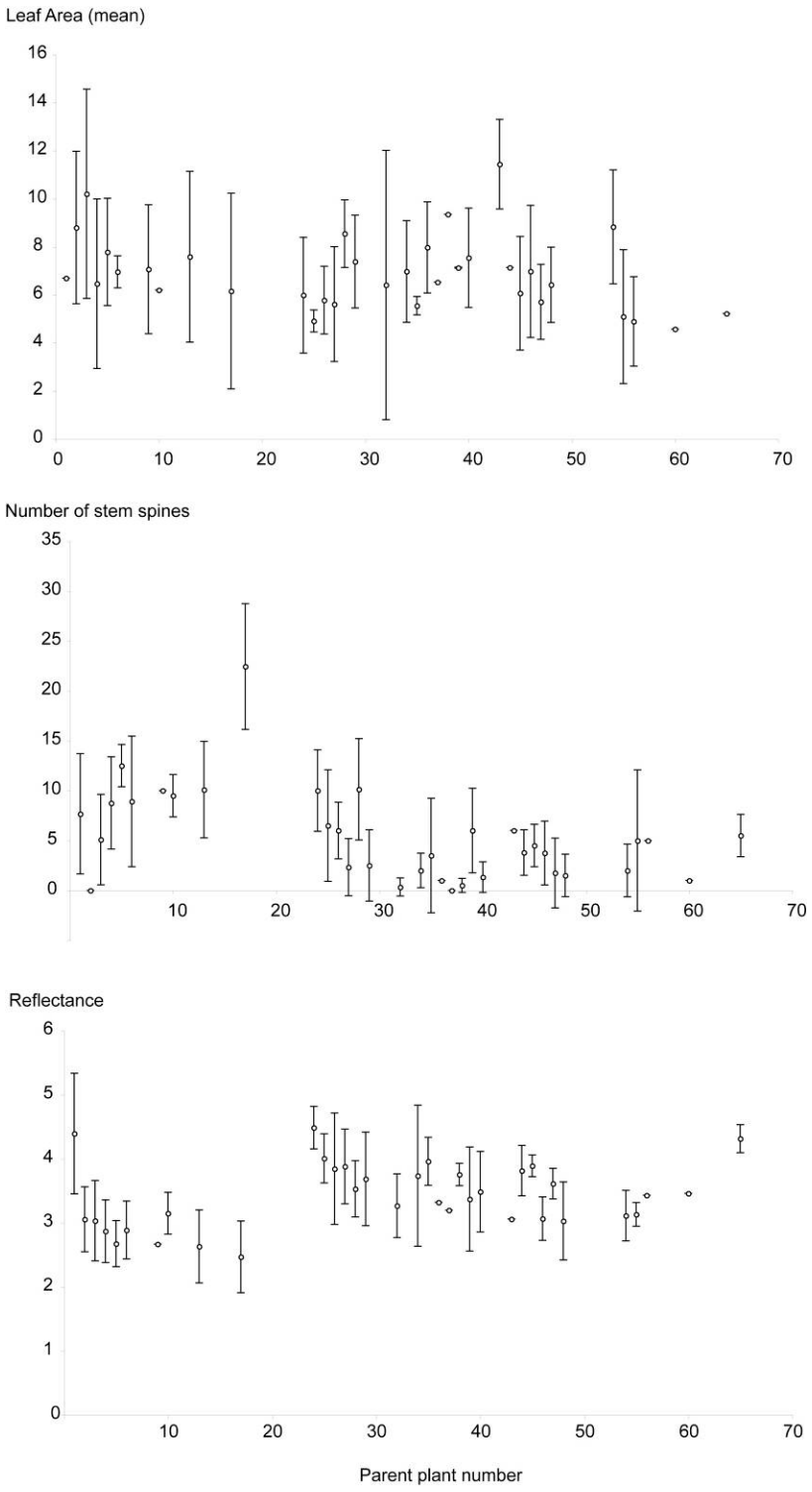

Figure 2.1 Morphometric variability within fruits for three measures. Each point represents the average data for all plants from a particular mother plant (i.e. fruits were collected from one plant), error bars are standard deviations. Note parent plant number 17 was *Solanum cleistogamum*, all others are *Solanum centrale*.

Experiment 2: Morphometric parameters varied considerably during the coarse of the study. Parameters measured included during the early stages of the experiment were: Leaf number, Total height, Largest leaf Length, Largest leaf width, Largest leaf area. At the end of the experiment, due to the high levels of variability across these parameters a total dry weight was used as an estimate of relative plant success. There were significant differences in overall plant biomass were detected between any nutrient addition and the

control (zero nutrient addition) (Figure 2.2, ANOVA; d.f.=2, F=228.57, p<0.0001). Fruit size had limited influence on total plant weight although small fruited plants differed from medium fruited plants overall (irrespective of nutrient addition) (Figure 2.3, ANOVA; d.f.=3, F=4.58, p<0.0036). There was no interaction between fruit size and nutrient treatment (Figure 2.4).

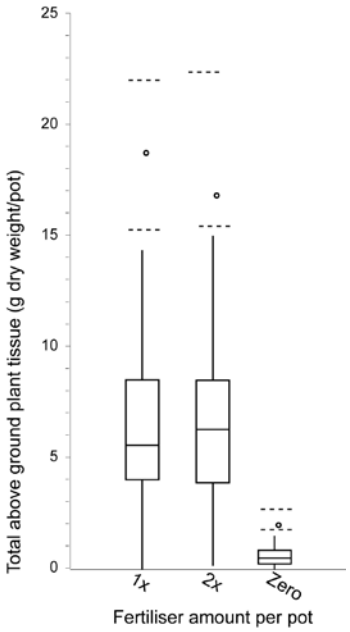

Figure 2.2 Box plot of total above ground biomass for three nutrient treatments, zero, 1X and 2X concentration, for *Solanum centrale* grown in the shadehouse at the Alice Springs Desert Park March-November 2009. Whiskers represent 25th and 75th percentiles, the box represents +/- one standard deviation from the mean, the line across the box represents the median and circles above the dotted line are outliers.

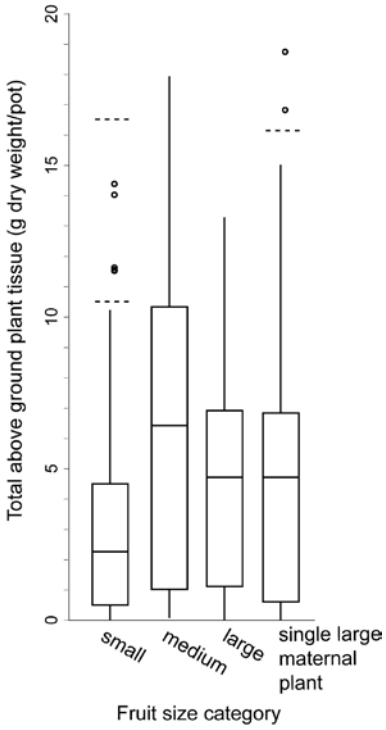

Figure 2.3 Box plot of total above ground biomass for four different fruit sizes, for *Solanum centrale* grown in the shadehouse at the Alice Springs Desert Park March-November 2009. Whiskers represent 25th and 75th

percentiles, the box represents +/- one standard deviation from the mean, the line across the box represents the median and circles above the dotted line are outliers.

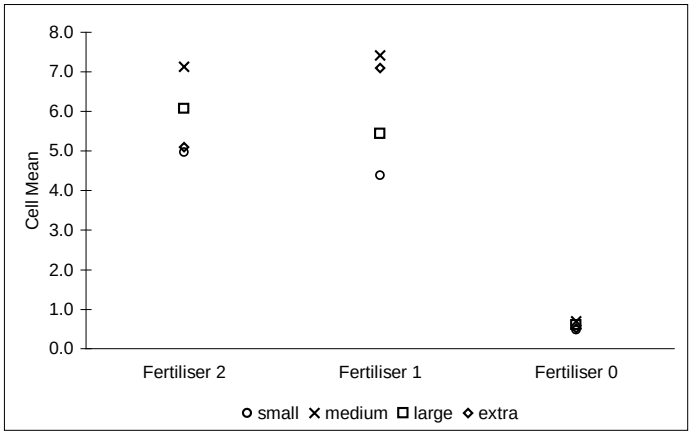

Figure 2.4 Plot of mean above ground biomass for three nutrient treatments, and four fruit sizes, for *Solanum centrale* grown in the shadehouse at the Alice Springs Desert Park March-November 2009.

The outcome of experiment two was that there is an obvious benefit of adding a standard nutrient treatment to growing *Solanum centrale* but that the addition of extra nutrients did not yield significantly improved biomass.

Morphological variation for total plant biomass was high (Figure 2.5), in agreement with analysis of seedling growth studies conducted under differing growth conditions at James Cook Universities growth facilities (Experiment 1) compared to the Alice Springs Desert Park (Experiment 2).

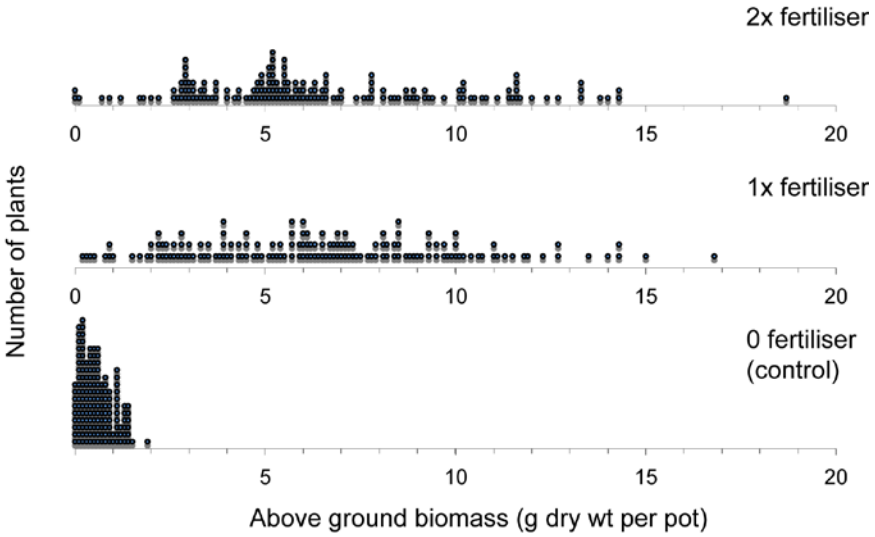

Figure 2.5 Plot depicting counts of biomass categories of above ground biomass for three nutrient treatments, for *Solanum centrale* grown in the shadehouse at the Alice Springs Desert Park March-November 2009.

Experiment 3: Growth trials of *Solanum centrale* from multiple fruits collected from the same large fruited maternal plant gave the same result for total plant biomass as obtained from numerous different maternal

plants where zero nutrients added was significantly different to both nutrient addition treatments (d.f.=2, $F=233.39$, $p<0.001$). The same result was obtained for measures of total plant height, total number leaves, and largest leaf area (all $p<0.001$). No significant differences were detected between the 1x and 2x nutrient additions.

The outcome of experiment three was that there is an obvious benefit of adding a standard nutrient treatment to growing *Solanum centrale* but that the addition of extra nutrients did not yield significantly improved biomass. Unfortunately, trials to assess the interactions between the reponses of plants to nutrient additions and the genotypes observed were unable to be tested.

Outcomes

- *Solanum centrale* has high levels of morphological variation irrespective of weather plants are derived from randomly collected fruits, fruits from limited genetic stock grown in horticultural trials in the AZRI plot, or fruits from the same maternal plant.
- Significant improvements to plant biomass, leaf number and plant height are gained from the addition of a standard dose of slow release fertiliser such as osmocote standard preparation (4.5g/L osmocote). Doubling osmocote concentration did not yield higher growth rates.
- Small fruits tend to yield plants with poorer growth performance.

Chapter 3a: Seed germination for horticulture

Proposed germination protocol for Horticulture

Michelle Waycott, Heather Robson, Ange Vincent and Tim Collins

Germination of *Solanum centrale* and other desert *Solanum* species is erratic if fruits are collected directly off plants and then used for direct seeding or for growing plants in plots. Experimental tests of germination responses to plant hormones and smoke water in different concentrations revealed that repeated treatment with 2.5% smoke water yielded very high germination results. The following germination protocol has been developed for small scale horticultural applications.

Materials

- Saturated smoke water (able to be purchased or made by bubbling smoke through water for several hours)
- Whole *Solanum* fruits, our trials have used seeds at least 3 years old, perhaps older from unknown sources
- Clean fungicide treated trays/plastic containers
- Fine domestic sieve 1-2 mm mesh
- Pump fine spray applicator

Protocol

- Dilute smoke water to 2.5% (i.e. 25 mL per L) with normal tapwater although boiled water would be preferable (anywhere in the range of 2.5-10% smoke water will be adequate, referred to hereafter as 'dilute smoke water')
- Place fruits in 2.5% smoke water solution for at least 12 hours, fruits should soften and the pulp become easy to separate out
- OPTIONAL
    - Remove seeds from pulp, this involves separating out the seeds onto paper towel or into another container of dilute smoke water using tweezers or forceps.
    - Seeds can be then handled more easily although it is very time consuming to do this and appears to not improve germination rates significantly although this has not been tested
- Mix seeds or seed/pulp mix well and plant out, spread seed at a rate assuming a 50% germination.
- Water seeds with the dilute smoke water weekly until germination of 50% has been achieved. This should be within 4 weeks of soaking the seeds. Seed beds should be kept moist and if likely to be attacked by fungus should be treated accordingly. Standard seedling treatment with broad spectrum fungicide or similar does not appear to impair germination although not specifically tested.

Caveat

- Seed sources vary considerably in their germinability. Wild collected fruits and fruits harvested from plants in cultivation yield between 5% and 95% germination rates.

Supplies details

Seed Raising Mix

By Rich Grow Garden Products

Ph-0894551323

Email-info@richgro.com.au

Smoke Water-Liquid Smoke Master

Purchased from Sure Gro

Phone-1800 643 384

Fax 0395580505

sales@suregro.com

Plant Food-Miracle-Gro All Purpose Water Soluble Plant Food

Manufactured by Scotts Australia Pty Ltd

Recommended rate of use 1 small scoop (1.25ml)/Litre but I used 1 small scoop/2L. See notes above on further dilution.

Chapter 3b: Draft manuscript to be submitted to horticulture journal, publication in preparation, awaiting partner agreements

Optimising Germination in Akatyerr, *Solanum centrale* and some other closely related *Solanum* species

Michelle Waycott[1,2], Heather Robson[1,2],Tim Collins[2,3] ,Simon Robson[1], & Maarten Ryder[2,4]

[1]*School of Marine & Tropical Biology, James Cook University, Townsville, QLD 4811.*

[2] *DKCRC.*

[3] *Alice Springs Desert Park, Alice Springs, NT 0871.*

[4] *CSIRO Sustainable Ecosystems, Glen Osmond SA 5064.*

Introduction

Akatyerr, like many other Australian native plant species, have highly variable seed germination (Johnson, Ahmed *et al.* 2003). A key component to the advancing our ability to undertake effective propagation for plantation growth of Akatyerr will be to improve germination of wild collected seeds. A major cause of erratic seed germination in native plants is dormancy, a strategy plants use to avoid periods of unsuitable climatic conditions. There are many commonly used dormancy-breaking techniques that promote germination in native species (Allan, Adkins *et al.* 2004). Some of these methods include fire and fire related products such as smoke water (Dixon, Roche *et al.* 1995). Other methods include the application of plant growth regulators such at gibberllic acid (Bell, Rokich *et al.* 1995).

The aims of this study were to:

- identify which dormancy breaking techniques increase germination rates in *Solanum* species,
- determine the optimal concentration of smoke-water to promote germination in *S. centrale*,
- assess if the germination success in *S. centrale* seeds was influenced by individual fruit characteristics, the broad geographic region from which the fruit originated.

Materials and Methods

Test of dormancy breaking techniques

The experimental species, the approximate age of their respective seeds and the number of seeds included in each experiment are as follows: *Solanum centrale* (1 – 4 yrs, 100 seeds), *S. chippendalei* (2 – 22 yrs, 50 seeds), *S. cleistogamum* (fresh, 100 seeds) and *S. quadriloculatum* (fresh, 100 seeds). Seeds were treated with one of the following treatments: control (demineralised water for 24 hrs), smokewater (10% for 24, 48 or 72 hrs), gibberellic acid (500 mg/L for 24 hrs), 0.1% potassium nitrate (KNO_3, for 24 hrs), or fire on top of punnet, then planted in standard nursery punnets and misted intermittently. *Solanum centrale* seeds were collected from Central, Western and Eastern Anmatyerr regions. Other species were collected from various

central desert regions. Germination was recorded by the emergence of the hypocotyl and the date at which final germination data was taken varied between species. Experiments were conducted at the Alice Springs Desert Park.

Test of smoke water concentration effects, fruit size, number and region effects

Solanum centrale seeds were obtained from wholesalers who estimated that they were from the Central, Western and Eastern Anmatyerr regions, from fruits between 1 and 4 yrs old (Western and Eastern) or less than 1 year old (Central). Sixteen fruits were randomly selected from each region. Fruit weight and the total number seeds per fruit were recorded. Five seeds were randomly selected from each fruit and individually raised under one of five smoke water treatments (0%, 2.5%, 5%, 10% & 20%) which was applied fortnightly. This experiment was replicated 4 times for a total of 960 growth trials. Seeds were reared in individual nursery punnets and germination was recorded by the emergence of the root from the seed coat. Treatments, plants and seeds were allocated in a randomized block design to avoid confounding position effects. Experiments were conducted at James Cook University.

Results

Test of dormancy breaking techniques

The percentage germination success of *Solanum centrale* was consistently highest under the gibberellic acid treatment, with the potassium nitrate appearing not to differ from that of the control treatment (Table 1).Germination success appears to vary with the region from which the seeds were collected, but it is not clear if the regional effect is consistent across all treatments. Seeds from Western Anmatyerr, for example, have a much higher germination success when treated with gibberellic acid than those from Central or Eastern Anmatyerr (66% vs. 25% & 16%), while seeds from the Eastern Anmatyerr appear to have a higher germination success than the other regions when treated with 10% smoke water (Table 1).

Table 1. Number of *S. centrale* seeds germinating from various Anmatyerr regions, 45 days post planting.

	Seed Treatments						
	Control 24 hrs	SW (10%) 24 hrs	SW (10%) 48 hrs	SW (10%) 72 hrs	GA 72 hrs	KNO3 24 hrs	Fire 2 min
Central Anmatyerr	0%	3%	3%	2%	35%	2%	7%
Western Anmatyerr	0%	8%	7%	12%	66%	1%	21%
Eastern Anmatyerr	1%	15%	11%	8%	16%	1%	14%

Smoke water, gibberellic acid and fire treatments appear to induce a higher germination success in *S. chippendalei*, *S. cleistogamum* and *S. quadriloculatum* than water only (Control) (Table 2).

Table 2. Percentage of seeds germinating for different *Solanum sp.* using either smoke water (SW) or gibberillic acid (GA). Germination success for *S. chippendalei*, *S. cleistogammum* and *S. quadriloculatum* seeds was recorded after 34, 14 & 28 days respectively.

		Seed Treatments		
Species	Accession	Control	SW (10%)	GA
S. chippendalei	RBB159	0%	-	11%
	A104026	0%	-	57%
S. cleistogamum	A102870	0%	54%	75%
S. quadriloculatum	A109786	0%	5%	48%

Test of smoke water concentration effects, fruit size, seed number and region effects

Solanum centrale germination success increased with the application of smoke water (ANOVA $F_{(4, 172)} = 17.3$, $p<0.001$, Fig. 1A), with germination success being similar for all concentrations above zero % (Tukeys post hoc test, $p>0.05$). There were also significant differences in germination rates between regions (ANOVA $F_{(2, 172)}=12.3$, $p=.00001$, Fig. 1B). Seeds from Western Anmatyerr showed the highest rate of germination followed by Eastern and then Central Anmatyerr. Overall germination rates approach 100% with successive additions of smoke water (Fig. 2).

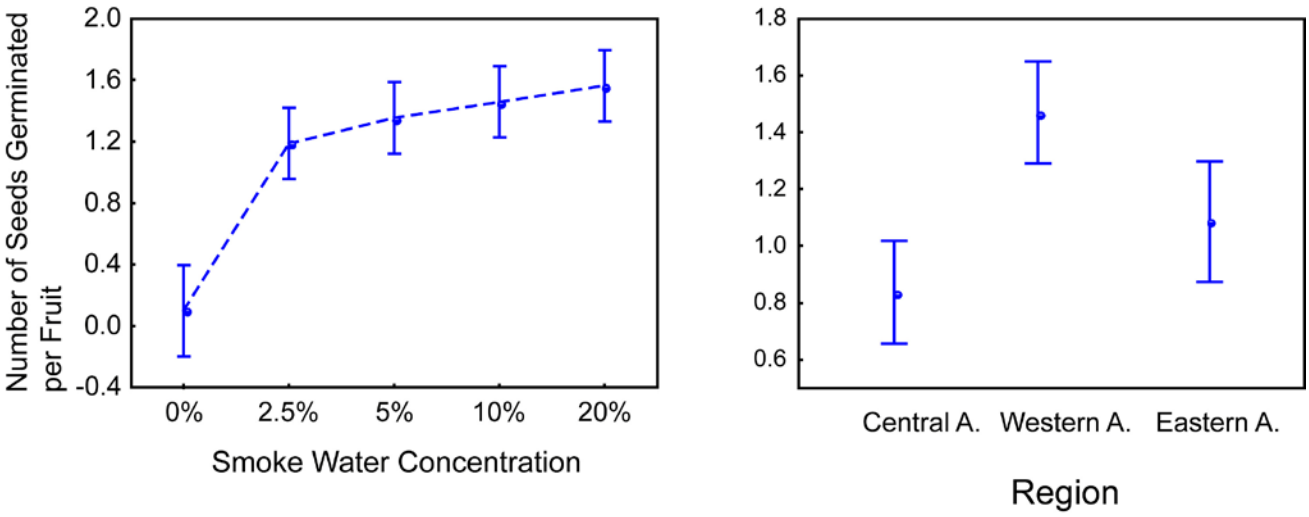

Fig 1. A (LHS) Effect of smoke water concentration on the and mean number of seeds germinating per fruit. Vertical bars equal 95% confidence intervals. B (RHS) Relationship between region of origin the mean number of seeds germination per fruit. Vertical bars equal 95% confidence intervals.

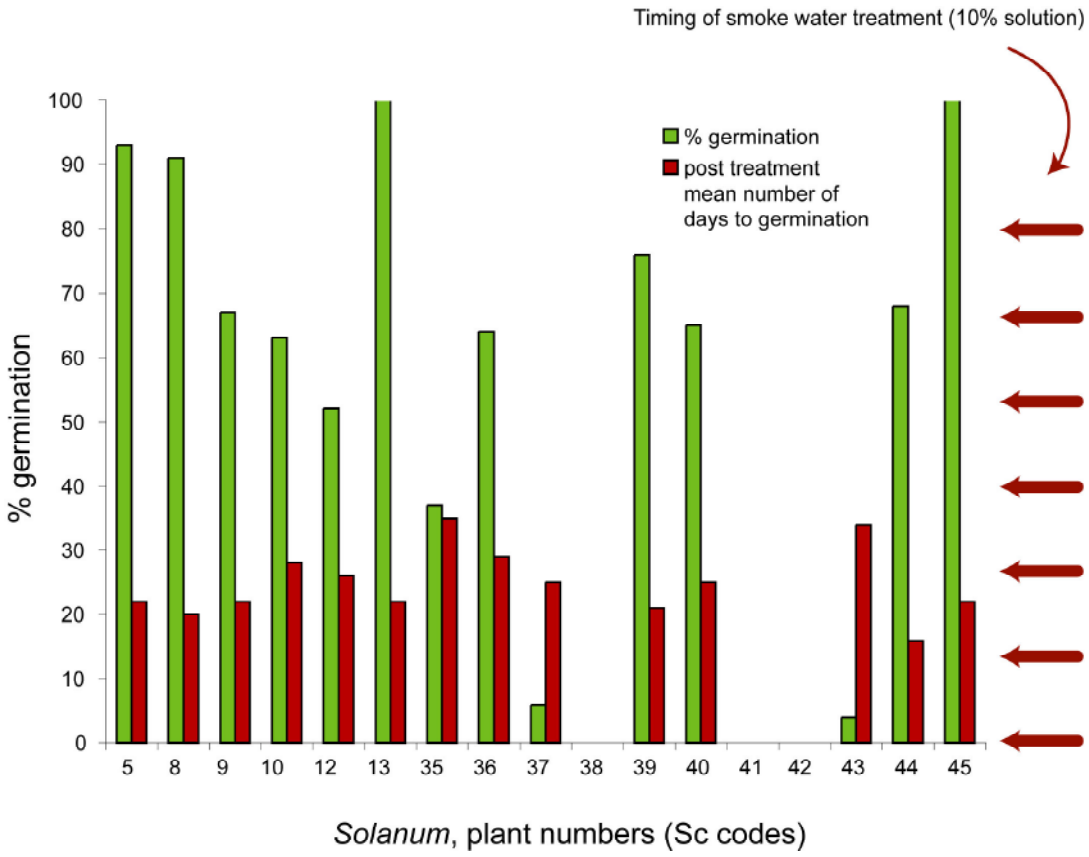

Fig 2. Effect of smoke water additions to % germination (cumulative) across seeds from all fruits used in the germination experiment.

There was a significant relationship between fruit weight and the number of seeds in a fruit when the data was pooled for all regions (Spearman's r = 0.65, p <0.001), this relationship is due in large part to the presence of larger fruits (with higher number of seeds) from the Western Anmatyerr region. When examined separately by region, there was no significant relationship between fruit size and seed number (Central Anmatyere: Spearmans r=0.02, p=0.65; Western Anmatyerr: r=0.51, p=0.11; Eastern Anmatyerr: r=0.47, p <0.12). Unfortunately the cause of difference among sampled regions cannot be attributed to a specific attribute; genetic, ecological, handling related or unknown factors. Material sourced from specific locations and known handling histories would improve our interpretability of any differences observed.

There was no significant relationship between the mean number of seeds germinating from a fruit and either the number of seeds within a fruit or the weight of the fruit (Spearman's r, p >0.05).

Outcomes

High rates of germination can be achieved using successive applications of smoke water. This may be commercially viable approach with some field modifications.

The germination rate of *S. centrale* differs between fruits from plants living in different regions. Germination success was greatest in *S. centrale* seeds collected from the Western Anmatyerr region which were also the larger fruits on average.

The application of smoke water, gibberellic acid or fire treatments increases the germination success of *Solanum centrale* seeds. It appears likely that these treatments also increase germination success in other species of *Solanum* e.g. *S. chippendalei, S. cleistogamum* and *S. quadriloculatum*.

The application of 2.5% smoke water induces a similar level of germinations success than does the application of 5%, 10% or 20% smoke water.

Within regions there is no relationship between the number of seeds in a fruit and the mass of the fruit, and no relationship between the germination success of seeds and either the number of seeds or the mass of the fruit from which they were collected. There was however, overall samples, a trend in fruit size and seed number indicating that regional differences in plant material may be important to yields and indicate good locations for sourcing larger fruited genetic stock for plant improvement. Specific experimental evidence would be required to assess regional and other differences in germinability and outcomes would benefit efforts to grow new plants from seed.

Summary and recommendations

Genetic diversity of *Solanum centrale* is high, individual plants potentially live for a long time as clones of *Solanum centrale* were detected on a scale of 10's of metres, possibly 100's of metres. Indeed, recruitment from seed may be very rare, the habit of this species being as a disturbance opportunist and thus plants may recruit in small windows of opportunity. The partitioning of genetic diversity is broadly associated with geographic regions and lends itself to potentially recognising regional provenances.

Polyploidy was detected in all wild population samples screened. The presence of polyploidy in *Solanum centrale* raises concerns about the genetic integrity of wild populations when plants of a different ploidy level are planted in horticultural plots. The Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act) aims to protect Australia's natural resources and genetic erosion in natural populations through careless horticultural practices threatens ecologically sustainable development of the fragile arid ecosystems that would be the target of this industry. Preliminary evidence that arid zone *Solanum* species may hybridise when they co-occur is of particular concern in the planting of any non-locally sourced material. It is recommended that a feasibility analysis of the impacts of genetic erosion of natural populations be conducted before further horticultural plantings are conduced. In addition, the distribution of polyploidy in arid zone *Solanum* species needs to be established as well as the propensity for Australian *Solanum* species to hybridise. Failing to heed these warnings may result in the production of sterile populations in the wild and the loss of valuable germplasm and/or destruction of the adaptability of this species. Although many other crop plants utilise polyploids in their plantings, extensive genetic evidence is utilised before attempting to potentially mix specifically selected polyploid plant lines and wild populations. This area of research may result in plant selections that could be appropriately planted alongside wild populations however it is unlikely to be a recommended approach until the detailed research has been conducted.

Simple techniques will improve the ability of arid zone *Solanum* to be put into agricultural settings. The addition of standard fertilisers, breaking dormancy using smoke water or its chemical constituents, and taking advantage of the growth form of this species are available to anyone cheaply and using readily available materials.

References (all sections)

Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. *Science* **294**, 321-326.

Ahmed AK, JohnsoN KA, Burchett MD, Kenny BJ (2006) The effects of heat, smoke, leaching, scarification, temperature and NaCl salinity on the germination of *Solanum centrale* (the Australian bush tomato). *Seed Science and Technology* **34**, 33-45.

Albrecht E, Escobar M, Chetelat RT (2010) Genetic diversity and population structure in the tomato-like nightshades *Solanum lycopersicoides* and *S. sitiens*. *Annals of Botany* **105**, 535-554.

Allan SM, Adkins SW, Preston CA, Bellairs SM (2004) Improved germination of the Australian natives: *Hibbertia commutate*, *Hibbertia amplexicaulis* (Dilleniaceae), *Chamaescilla corymbosa* (Liliaceae), and *Leucopogon nutans* (Epacridaceae). *Australian Journal of Botany* **52**, 345-351.

Bataillon TM, David JL, Schoen DJ (1996) Neutral genetic markers and conservation genetics: simulated germplasm collections. *Genetics* **144**, 409-417.

Bell D, Rokich D, McChesney C, Plummer J (1995) Effects of temperature, light, and gibberellic acid on the germination of seeds of 43 species native to Western Australia. *Journal of Vegetation Science* **6**, 797-806.

Benor S, Zhang MY, Wang ZF, Zhang HS (2008) Assessment of genetic variation in tomato (*Solanum lycopersicum* L.) inbred lines using SSR molecular markers. *Journal of Genetics and Genomics* **35**, 373-379.

Bornet B, Branchard M (2001) Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter* **19**, 209-215.

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *Biotechniques* **31**, 24-28.

Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics* **13**, 115-155.

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology* **13**, 2101-2106.

Bussell JD, Waycott M, Chappill JA (2005) Arbitrarily amplified DNA markers as characters for phylogenetic inference. *Perspectives in Plant Ecology Evolution and Systematics* **7**, 3-26.

Byrne M (1996) Application of DNA markers to the genetics of the Australian flora. In 'Gondwanan Heritage: past, present and future of the Western Australain Biota'. (Eds SD Hopper, JA Chappill, MS Harvey and AS George) pp. 259-270. (Surrey Beatty and Sons Pty. Limited: Chipping Norton)

Dixon K, Roche S, Pate J (1995) The promotive effect of smoke derived from burnt native vegetation on seed germination of Western Australian Plants. *Oecologia* **101**, 185-192.

Frary A, Xu YM, Liu JP, Mitchell S, Tedeschi E, Tanksley S (2005) Development of a set of PCR-based anchor markers encompassing the tomato genome and evaluation of their usefulness for genetics and breeding experiments. *Theoretical and Applied Genetics* **111**, 291-312.

Freas KE, Kemp PR (1983) Some relationships between enviromental reliability and seed dormancy in desert annual plants. *Journal of Ecology* **71**, 211-217.

Garza JC, Wlliamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* **10**, 305-318.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. *Molecular Evolution: Producing the Biochemical Data, Part B* **395**, 202-222.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques* **27**, 500-507.

Jiang J, Wang HH, Liu SA, Zhao XM (2009) 'Genetic diversity of tomato germplasm reveaed by SSR markers.'

Johnson K, Ahmed A, Armstrong G (2003) Investigations into in vitro manipulation of *Solanum centrale* (Bush Tomato). In: Proc. 1st IS on Accl. & Estab. Micropop. Plants Eds. A.S. Economou & P.E. Read. *Acta Horticulturae* **616**, 169-175.

Kadmon R (1993) Population dynamic consequences of habitat heterogeneity - an experimental-study. *Ecology* **74**, 816-825.

Lowe A, Harris S, Ashton P (2004) 'Ecological Genetics: Design, Analysis, and Application.' (Blackwell: Oxford UK)

Moon HS, Nifong JM, Nicholson JS, Heineman A, Lion K, van der Hoeven R, Hayes AJ, Lewis RS (2009) Microsatellite-based analysis of Tobacco (*Nicotiana tabacum* L.) genetic resources. *Crop Science* **49**, 2149-2159.

Moran GF, Butcher PA, Glaubitz JC (2000) Application of genetic markers in the domestication, conservation and utilisation of genetic resources of Australasian tree species. *Australian Journal of Botany* **48**, 313-320.

Nagaraju J, Kathirvel M, Subbaiah EV, Muthulakshmi M, Kumar LD (2002) FISSR-PCR: a simple and sensitive assay for highthroughput genotyping and genetic mapping. *Molecular and Cellular Probes* **16**, 67-72.

Nunome T, Negoro S, Kono I, Kanamori H, Miyatake K, Yamaguchi H, Ohyama A, Fukuoka H (2009) Development of SSR markers derived from SSR-enriched genomic library of eggplant (*Solanum melongena* L.). *Theoretical and Applied Genetics* **119**, 1143-1153.

Nunome T, Suwabe K, Iketani H, Hirai M (2003a) Identification and characterization of microsatellites in eggplant. *Plant Breeding* **122**, 256-262.

Nunome T, Suwabe K, Ohyama A, Fukuoka H (2003b) Characterization of trinucleotide microsatellites in eggplant. *Breeding Science* **53**, 77-83.

Otto SP, Whitton J (2000) Polyploid incidence and evolution. *Annual Review of Genetics* **34**, 401-437.

Queller DC, E SJ, Hughes CR (1993) Microsatellites and Kinship. *Trends in Ecology and Evolution* **8**, 285-288.

Randell BR, Symon DE (1976) Chromosome number in Australian *Solanum* species. *Australian Journal of Botany* **24**, 369-379.

Ritland K (2000) Marker-inferred relatedness as a tool for detecting heritability in nature. *Molecular Ecology* **9**, 1195-1204.

Robins J, Ryder M (2008) Bush tomato. In 'The New Crop Industries Handbook—Native Foods, Rural Industries Research and Development Corporation' pp. 346-352: Canberra, Australia)

Rosa PM, de Campos T, de Sousa ACB, Sforca DA, Torres GAM, de Souza AP (2010) Potato cultivar identification using molecular markers. *Pesquisa Agropecuaria Brasileira* **45**, 110-113.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In 'Bioinformatics Methods and Protocols: Methods in Molecular Biology'. (Ed. S Misener) pp. 365-386. (Humana Press: Totowa. NJ)

Ryzhova NN, Martirosyan EV, Kochieva EZ (2010) Analysis of microsatellite locus polymorphism in potato (*Solanum tuberosum*) cultivars of Russian breeding. *Russian Journal of Genetics* **46**, 425-430.

Scheiner SM (1993) Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* **24**, 35-68.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**, 615-629.

Sharma S, Sarkar D, Pandey SK (2010) Phenotypic characterization and nuclear microsatellite analysis reveal genomic changes and rearrangements underlying androgenesis in tetraploid potatoes (*Solanum tuberosum* L.). *Euphytica* **171**, 313-326.

Stagel A, Portis E, Toppino L, Rotino GL, Lanteri S (2008) Gene-based microsatellite development for mapping and phylogeny studies in eggplant. *Bmc Genomics* **9**.

Tautz D (1989) Hyperariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acid Research* **17**, 6463-6471.

Taylor AC, Horsup A, Johnson CN, Sunnucks P, Sherwin B (1997) Relatedness structure detected by microsatellite analysis. *Molecular Ecology* **6**, 9-19.

Wolfe AD, Xiang QY, Kephart SR (1998) Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. *Molecular Ecology* **7**, 1107-1125.

Young AG, Murray BG (2000) Genetic bottlenecks and dysgenic gene flow into re-established populations of the grassland daisy, *Rutidosis leptorrhynchoides*. *Australian Journal of Botany* **48**, 409-416.