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Rapid Identification of *Acacia* Species With Potential Salt Tolerance by Using Nuclear Ribosomal DNA Markers

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Abstract

Use of saline lands for agroforestry relies primarily on plant species that have the trait of salinity tolerance, and also other economic and agronomic benefits. The selection of species, however, also needs to consider other key factors such as compatibility with existing flora, and potential for environmental benefits such as improved soil fertility or lowering of the water table in the case of dryland salinity. The testing of candidate species in particular environments needs substantial investments of costs and time. In this paper, a novel approach is presented for the rapid identification of potentially salt tolerant *Acacia* species, based on molecular phylogenetic analysis. The approach has been applied to four species groups, *Acacia pendula*, *A. salicina*, *A. victoriae* and *A. stenophylla*. The nuclear-encoded ribosomal DNA internal and external transcribed spacer (ITS and ETS) regions were used as markers, and phylogenetic analyses undertaken to identify closely related species that may share the salt tolerance traits. Such a methodology could be used to more rapidly identify candidate native species for agroforestry in salinity-affected regions and for preventing further expansion of salinity, thus assisting in biodiversity conservation.

Keywords: *Acacia*, phylogeny, ribosomal RNA, ITS, ETS, salinity

Abbreviations: ETS: External Transcribed Spacer; IGS: Inter-Genic Spacer; ITS: Internal Transcribed Spacer; LSU: Large Sub-Unit; SSU: Small Sub-Unit.

1. Introduction

The environmental challenge of dryland salinity currently affects approximately 3.3 million hectares of arable lands in Australia, and could further expand to 5.7 million hectares if left unchecked (Department of Sustainability, Environment, Water, Population and Communities; <http://www.environment.gov.au/land/pressures/salinity/>). The negative impact of salinity has prompted serious action by the Australian government to implement programs such as the National Action Plan for Salinity and Water Quality (Pannell & Roberts, 2010). The Australian landscape has vast reserves of salt beneath the land surface. Species of perennial Australian native vegetation are well-adapted to these harsh conditions, utilizing available water, also maintaining the water table and immobilizing the salt. However, dramatic changes in land use since the European settlement have led to replacement of the deep-rooted native perennials with shallow-rooted cereal crops and pastures. This has resulted in mobilization of salt to the soil surface, causing toxicity to plants and significant loss of crop land and native vegetation. One of the key strategies for reclamation of salinity-degraded lands is revegetation with well-adapted native species, some of which maybe additionally useful as sources of food, fodder, fuel, fiber, resins, essential oils or pharmaceutical products (Maslin & McDonald, 2004).

The genus *Acacia* (wattle), first described in 1754 by Philip Miller, belongs to the tribe Acacieae and is the

largest genus within the legume subfamily *Mimosoideae* (Murphy, 2008). The name *Acacia* is now restricted to the subgenus formerly known as *Phyllodineae* (synonym *Racosperma*), confining it mainly to Australia (Maslin, 2006), where it is the largest genus of vascular plants and widespread in dry and semi-arid regions. *Acacia* species thrive in a diverse range of environments and many species tolerate high salinity, sodicity, high pH and waterlogged soils (Niknam & McComb, 2000). Additionally, several salt-tolerant acacias, such as *A. saligna*, *A. stenophylla*, *A. salicina* and *A. ampliceps*, have the potential to provide forage and fodder (Vercoe, 1987). *Acacia* species harbor nitrogen-fixing rhizobia that can improve soil fertility (Hoque, Broadhurst, & Thrall, 2011) and are in use for reclamation of severely degraded lands in Brazil (Chaer, Resende, Campello, De Faria, & Boddey, 2011). Some species provide timber and edible fruit and seeds. However, despite the benefits, *Acacia* species are currently under-investigated and underutilized, and information on salinity tolerant species with agroforestry potential is especially limited. Four species (*A. pendula*, *A. salicina*, *A. stenophylla*, *A. victoriae*), were found to be salt tolerant (among other vegetation) in a unique project on reclamation of saline lands in Victoria (Australia), [Phil Dyson and Ian Rankin, Northern United Forestry Group (NUFG) Bendigo, Australia; personal communications 2009] areas of which show high surface soil salinity [12-14 deci-Siemens/metre (dS/m)] and sub-soil salinity (4 dS/m) (NUFG Kamarooka Project; <http://www.nufg.org.au/Kamarooka%20Project.htm>). This study aims to use molecular phylogenetics to rapidly identify the close relatives of these species in order to exploit their potential for agroforestry on saline lands.

Nuclear ribosomal DNA (rDNA) markers such as the internal transcribed spacer (ITS) and the external transcribed spacer (ETS) (Baldwin & Markos, 1998; Mort et al., 2007) and chloroplast loci (e.g. *atpF-H*, *matK*, *psbK-I*, *rbcL*, *rpoB*, *rpoC1* and *trnH-psbA*) (Dong, Liu, Yu, Wang, & Zhou, 2012) have been commonly used in plant molecular systematics. The ITS1 and ITS2 regions of plant rRNA genes are used most frequently to study phylogeny at genus and species levels due to advantages such as high sequence variability, copy numbers and ease of amplification (Kay, Whittall, & Hodges, 2006; Mort et al., 2007). ITS is adjacent to the conserved 5.8S rRNA gene region and is flanked by the conserved 18S and 26S rRNA genes, and this entire region is tandemly repeated thousands of times to make up the rDNA. The ETS of 18S-26S rDNA belongs to the same transcriptional unit. It may have evolved under similar functional constraints and complements the ITS data to yield more characters for significant phylogenetic inferences in angiosperms (Baldwin & Markos, 1998). The ITS and ETS are currently the most commonly sequenced loci for *Acacia* and provide the best available comparative dataset.

Comparative biology is based on the expectation that closely related organisms share traits, such as salinity tolerance, that are less common in more distantly related organisms (Cracraft, 2002). Therefore, clarifying molecular phylogenetic relationships can aid in selecting candidate species for a particular trait. Miller, Murphy, Brown, Richardson, and González-Orozco (2011) used plastid and nuclear rDNA data to test for invasiveness of species across a broad framework of 110 acacias. Although the invasive species did not form a monophyletic group, some evidence for phylogenetic grouping of invasive species was found. The study also identified sister species of the known invasive species that may have increased potential for invasiveness. The present study takes a similar approach, to rapidly identify sister species of known salt tolerant taxa. ITS and ETS sequence data were obtained for species in morphological groups closely related to the four salt-tolerant species, *A. pendula*, *A. salicina*, *A. stenophylla* and *A. victoriae*, mentioned above. These data, combined with an extensive *Acacia* dataset of ITS and ETS markers, was used to generate phylogenetic relationships and identify further species with a potential for salt tolerance.

2. Materials and Methods

2.1 Plant Tissue Sampling for Genomic DNA Extraction

The thirty species to be analysed were initially selected based on species groups morphologically related to the salt tolerant species described above (*A. pendula*, *A. salicina*, *A. stenophylla* and *A. victoriae*), as described in Flora of Australia (Orchard & Wilson, 2001a; b). The phyllode or leaf tissue (20mg) was removed from the herbarium sheets held at Royal Botanic Gardens, Melbourne (herbarium voucher numbers given in Table 1) and used for genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen Australia). A dataset of ITS and ETS sequences was constructed using data from Brown, Murphy, Kidman, and Ladiges (2012). *Paraserianthes lophantha* (voucher MEL2057862; GenBank accessions: ITS: EF638203; ETS: EF638105.1) was used as the out-group, based on Brown, Murphy, Miller, and Ladiges (2008) who concluded that it is sister to *Acacia*.

Table 1. Identification of species morphologically related to *A. stenophylla*, *A. salicina*, *A. pendula* and *A. victoriae*

Morphologically related species	Key indicator(s) of plausible relationship	References	Voucher number
Species of interest: <i>A. stenophylla</i>			MEL 2312528
<i>A. coriacea</i>	Long phyllodes and growth habit resembling <i>A. stenophylla</i> .	2	MEL 2313071
<i>A. sibilans</i>	Closely related to <i>A. coriacea</i> in phyllode morphology.	2	MEL2327552
<i>A. calcicola</i>	<i>A. coriacea</i> sometimes confused with <i>A. calcicola</i> .	2	MEL 2233895
Species of interest: <i>A. salicina</i>			MEL 2286869
<i>A. bivenosa</i>	<i>A. salicina</i> belongs to the <i>A. bivenosa</i> group.	3	MEL 2306646
<i>A. ligulata</i>	<i>A. salicina</i> was often confused with <i>A. ligulata</i> and <i>A. ampliceps</i> .	1, 3	MEL 2326226
<i>A. ampliceps</i>	<i>A. salicina</i> was often confused with <i>A. ligulata</i> and <i>A. ampliceps</i> .	1, 3	MEL 2306643
<i>A. cupularis</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2278496
<i>A. didyma</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2283488
<i>A. rostellifera</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2319042
<i>A. sclerosperma</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2042807
<i>A. startii</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2297013
<i>A. telmica</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 710783
<i>A. tysonii</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2137099
<i>A. xanthina</i>	Belongs to the <i>A. bivenosa</i> group.	3	MEL 2327555
Species of interest: <i>A. pendula</i>			MEL 2233883
<i>A. omalophylla</i>	<i>A. pendula</i> closely related to <i>A. omalophylla</i> .	2	MEL 2328341
<i>A. melvillei</i> *	Very closely related to <i>A. omalophylla</i> , and in Queensland, both are known as Yarran.	2	MEL 2034608
Species of interest: <i>A. victoriae</i> *			MELU SRA 260
<i>A. alexandri</i> *	<i>A. victoriae</i> with long and linear phyllodes may be confused with <i>A. alexandri</i> .	2, 4	MELU SRA 148
<i>A. aphanoclada</i> *	Belongs to the <i>A. victoriae</i> group.	2, 4	MELU SRA 224
<i>A. chartacea</i>	Belongs to the <i>A. victoriae</i> group.	2, 4	MEL 721448
<i>A. cuspidifolia</i> *	Belongs to the <i>A. victoriae</i> group.	2, 4	MEL SRA 115
<i>A. dempsteri</i> *	Belongs to the <i>A. victoriae</i> group.	2, 4	MEL 2096892
<i>A. pickardii</i>	Belongs to the <i>A. victoriae</i> group.	2, 4	MEL 2067966
<i>A. ryaniana</i>	Belongs to the <i>A. victoriae</i> group.	2, 4	MEL 721629
<i>A. synchronicia</i>	In the absence of flowers, <i>A. victoriae</i> may be confused with <i>A. synchronicia</i> .	2, 4	MEL 2252506
<i>A. marramamba</i>	Belongs to the <i>A. pyrifolia</i> group.	2, 4	MEL 2313077
<i>A. stronglyphylla</i>	Presence of spinose stipules.	2, 4	MEL 2287670

*Sequences available in GenBank: *A. melvillei*: FJ868397.1; FJ868438.1; *A. victoriae*: DQ029275.1, DQ029316.1; *A. alexandri*: DQ029264.1, DQ029306.1; *A. cuspidifolia*: DQ029261.1, DQ029302.1; *A. dempsteri*: DQ029259.1, DQ029300.1. References: 1: Maslin (2001); 2: Orchard & Wilson (2001b); 3: Chapman & Maslin (1992); 4: Ariati et al. (2006).

2.2 Amplification and Sequencing of ITS and ETS Markers

The ITS region was amplified from the genomic DNAs using the primer pair S3 (5'-AACCTGCGGAAGGATCATTG-3') and 26SE (5'-TAGAATCCCCGGTTCGCTCGCCGT-3') as described previously (Murphy, Miller, Bayer, & Ladiges, 2003). The ETS region was amplified using the primer pair 18S-IGS (5'-CACATGCATGGCTTAATCTTTG-3') and AcR2 (5'-GGGCGTGTGAGTGGTGTGTTGG-3'), as per Murphy, Brown, Miller, and Ladiges (2010) (Figure 1). The 50 μ L reaction mixes contained 2mM dNTPs, 25 pmol of each primer, 10-50 ng of DNA template, 1U *Taq* DNA polymerase and 5 μ L 10 X reaction buffer (New England Biolabs, Madison, USA). The PCR products were purified using QIAquick PCR kit (Qiagen) and used for DNA sequencing in both directions for each sample, using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit or Prism Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Mulgrave, Australia) and the supplier's protocols.

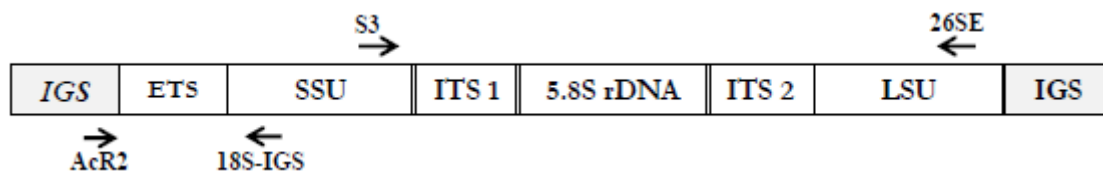


Figure 1. Structure of the ribosomal DNA cistron

S3 denotes the forward primer and 26SE denotes the reverse primer used to amplify the ITS region. AcR2 denotes the forward primer and 18S-IGS denotes the reverse primer used to amplify the ETS region (figure not drawn to scale).

2.3 Phylogenetic Analysis

The sequences generated in this study were edited using Sequencher v3.0 (Gene Codes Corporation) and concatenated manually using BioEdit v7.0.0 (Hall, 2007), followed by alignment using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The sequence dataset obtained for 19 species was combined with previous data (Brown et al., 2012) and subjected to Bayesian analysis using MrBayes v3.2.1 (Ronquist & Huelsenbeck, 2003). For analysis, the combined ITS and ETS data were divided into six partitions: ITS1, 5.8S, ITS2, LSU, SSU and ETS. Insertion/deletion (indel) events were scored as multistate characters. An evolutionary model, GTR (Generalised Time Reversible) substitution model with gamma-distributed rate variation, was applied to each partition. A Markov Chain Monte Carlo (MCMC) search was run for 8 million generations, with trees sampled every 100 generations. Starting from different random trees, the analyses were performed twice simultaneously (Nruns = 2) with four Markov chains (N chains = 4) for each tree. Burn-in was set to 25001 (i.e. the first 25001 trees were discarded from each run). A Bayesian consensus phylogram was generated and for each node posterior probability (PP) values were calculated. The phylogenetic tree was visualised and coded for display using FigTree (Rambaut & Drummond, 2008).

3. Results

3.1 Sequence Data

Of the 30 species of interest sampled based on morphological relatedness, 19 yielded good quality sequence data for both ITS and ETS markers. For five species (*A. victoriae*, *A. alexandri*, *A. aphanoclada*, *A. cuspidifolia*, *A. dempsteri*) that did not produce high quality sequences, the ITS and ETS data available in GenBank as part of the dataset of Brown et al. (2012) were used for further analysis. Six species (*A. ampliceps*, *A. bivenosa*, *A. didyma*, *A. sclerosperma*, *A. startii*, *A. telmica*) could not be analysed due to poor amplification results or poor quality sequences, and there were no corresponding sequences in GenBank; hence these were excluded from further analysis. The length of the individual regions, including the aligned versus the unaligned (raw sequence) lengths, for the 24 species is given in Table 2. The partial length of ITS1 (196 base pairs; bp) (Table 2) appears somewhat shorter than previously reported lengths for *Acacia* ITS1 (Murphy et al., 2003; 2010), due to some unresolved bases occurring close to the primer binding site near the 5' end of the ITS. The sequence data for 5.8S subunit and ITS2 region were complete. All 5.8S subunit sequences were 159 bp long, as found previously (Murphy et al., 2003; 2010). The ITS2 ranged from 149 bp (*A. pickardii*) to 215 bp (*A. xanthina*). The ETS region varied from 393 to 407 bp.

Table 2. Sequence characteristics of ITS1, 5.8S, ITS2 and ETS regions

Sequenced region	ITS1 (1-196)	5.8S (197-355)	ITS2 (356-571)	LSU (572-712)	ETS (713-1129)	SSU (1130-1147)
Aligned length	196	159	216	141	416	18
Unaligned length	108-188	159	149-215	0-137	393-407	17-18

Numbers indicate length in bp. The aligned length indicates an overall range of lengths, based on the alignment of all individual raw sequences and including gaps. The unaligned length denotes the lengths of raw sequences from individual species without gaps.

3.2 Bayesian Analysis of the Combined ITS and ETS Regions Incorporated Into the Larger Dataset

The sequence data generated for the 24 taxa altogether were incorporated into the larger dataset of Brown et al. (2012) for a total of 178 sequences (including the out-group *P. lophantha*) and used for Bayesian analysis. The length of the concatenated ITS and ETS sequences was 1290 bp. A total of 8 indel characters (6 from ITS and 2 from ETS regions) were scored. The average standard deviation of split frequencies was 0.010 and the average potential scale reduction factor was 1.001. The consistency index was 0.146 and retention index was 0.695. Incorporation of the new dataset showed that some of the species fall into previously recognised clades (Murphy et al., 2010; Miller et al., 2011; Brown et al., 2012), e.g., the *A. aulacocarpa* group, *A. auriculiformis* group, and the *A. victoriae* and *A. pyriformis* group (Figure 2).

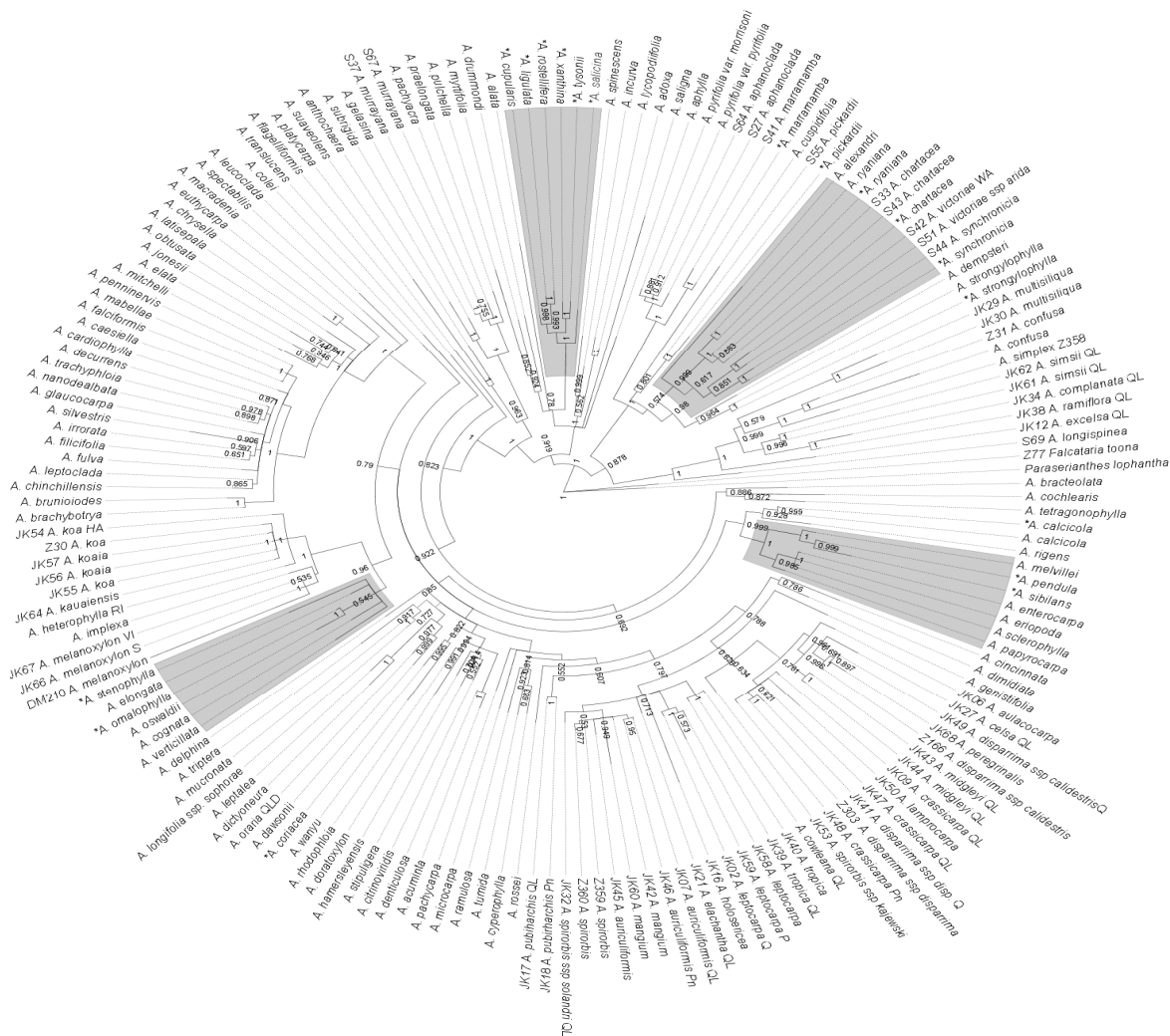


Figure 2. Phylogenetic tree of *Acacia* species

The tree is displayed using Fig Tree (Rambaut and Drummond, 2008), with data from Bayesian analysis of 178 *Acacia* species. The four known salinity-tolerant species of interest (*A. victoriae*, *A. salicina*, *A. pendula*, *A. stenophylla*) and their closest relatives are shaded in grey.

As seen in Figure 2, *A. pendula* is sister to *A. sibilans*, and *A. stenophylla* falls into a weakly supported (PP = 0.545) clade comprising *A. omalophylla*, *A. elongata*, *A. oswaldii*, *A. cognata* and *A. verticillata*. This observation varies slightly from that based on morphological similarity; *A. pendula* was expected to be more related to *A. omalophylla*, and in turn to *A. melvillei*, and *A. sibilans* is morphologically related to *A. stenophylla* as per Flora of Australia (Orchard & Wilson, 2001b). *A. pendula* sampled in this study is closely related to taxa in two smaller clades (*A. papyrocarpa*, *A. enterocarpa*, *A. eriopoda* and *A. sclerophylla*, and *A. calcicola* and *A. rigens*). This group has narrow, sclerophyllous phyllodes and grows mostly in arid and semi-arid regions (Murphy et al., 2010). The results of the present study suggest that further morphological and molecular sampling of these groups is necessary, as specimens in herbaria may be cryptic and misidentification cannot be ruled out for non-fruiting material.

The *A. victoriae* group, which is named after the taxon *A. victoriae*, is closely related to the *A. pyrifolia* group, and the two groups were informally named as the '*A. victoriae* and *A. pyrifolia* clade' by Murphy et al. (2010). In this study, *A. victoriae* is found to be sister to *A. synchronicia*, and in turn is placed in a larger clade with *A. chartacea*, *A. ryaniana* and *A. alexandri*. This strongly supported clade (PP = 0.999) has been resolved in previous reports (Ariati, Murphy, Udovicic, & Ladiges, 2006; Murphy et al., 2010; Miller et al., 2011; Brown et al., 2012), but the salinity tolerance of these species has not been assessed.

The fourth grouping resolved *A. salicina* in a strongly supported clade (PP=1.0) with *A. cupularis*, *A. ligulata*, *A. rostellifera*, *A. xanthina* and *A. tysonii*, as expected based on their morphological grouping as the '*A. bivenosa*' group, as per Flora of Australia (Orchard & Wilson; 2001a; b). However, *A. cupularis* is sister to *A. ligulata* and *A. rostellifera* than to *A. xanthina* as predicted.

4. Discussion

In this paper, the development of new ITS and ETS molecular datasets for 19 taxa of specific interest due to their close relation to four known salt-tolerant species is reported. This is followed by incorporation of these samples into a larger molecular phylogenetic study of Brown et al. (2012). The paper serves as a model for more efficient use of molecular data not only for testing phylogenetic relationships, but also for other application-oriented outcomes.

The four main salt-tolerant species, *A. pendula*, *A. stenophylla*, *A. salicina* and *A. victoriae*, selected based on the Kamarooka Project (NUFG, Bendigo, Australia; <http://www.nufg.org.au/Kamarooka%20Project.htm>) for this work, have other potential applications, but are currently under-utilized. *A. pendula* is known for its strong and dense wood, and used as an ornamental tree, or for firewood, and for making small wooden articles (Department of Primary Industries, Victoria; <http://www.dpi.vic.gov.au/forestry/investment-trade/regional-information/farm-forestry-in-the-north-central-region/myall>). *A. pendula* is also a useful stock fodder in drought (World Wide Wattle; <http://www.worldwidewattle.com/>). Some species related to *A. pendula* are salt tolerant, e.g., *A. calcicola*, with a salinity tolerance of 16.5 dS/m under hydroponic conditions (Malik & Ahmed, 2002) and 19.9 dS/m of root zone salinity (Akhter, Ahmed, & Malik, 2002), while others such as *A. papyrocarpa* may be suitable for salty land (Government of South Australia, http://www.pir.sa.gov.au/__data/assets/pdf_file/0005/139163/salinity.pdf), but a definitive range of their tolerance is yet to be determined. *A. calcicola*, with a tolerance range of 16.5-19.9 dS/m, can be classified as highly salt tolerant, with soil electrical conductivity (EC) >16 dS/m being considered extremely saline (Food and Agricultural Organisation (FAO) <http://www.fao.org/docrep/x5871e/x5871e04.htm>). Additionally, *A. calcicola* can be used for fuel, shade, shelter and windbreaks (FAO; <http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=2591>). *A. enterocarpa*, another close relative, is a nationally 'Endangered' species (Moritz & Bickerton, 2011), and both species have been reported as frost tolerant (Bird, Kearney, & Jowett, 1996). Thus certain favourable characteristics may be shared within clades identified in phylogenetic studies.

A. stenophylla falls into a weakly supported clade with *A. verticillata*, *A. cognata*, *A. elongata*, *A. oswaldii* and *A. omalophylla* (Figure 2), although the relationships within this clade are not fully resolved. *A. stenophylla* is capable of growth in extremely saline sites (EC >16 dS/m) (FAO). It is highly frost tolerant (Bird et al., 1996) as well as tolerant to highly alkaline soils and waterlogging (Marcar & Crawford, 2004). The salinity tolerance of *A. verticillata*, *A. elongata*, *A. oswaldii* and *A. omalophylla* needs to be determined.

The *A. victoriae* group is a well-characterised monophyletic clade (Ariati et al., 2006). Maslin (1992) had revised this group and included ten species (nine included in the present analysis) found mainly in arid parts of Australia, i.e., *A. victoriae* Benth., *A. alexandri* Maslin, *A. aphanoclada* Maslin, *A. chartacea* Maslin, *A. cuspidifolia* Maslin, *A. dempsteri* F. Muell., *A. glaucocaesia* Domin, *A. pickardii* Tind., *A. ryaniana* Maslin and *A. synchronicia* Maslin. Most of these are characterised by spinose stipules, similar phyllode characteristics and a minute gland near the apical mucro (Ariati et al., 2006; Orchard & Wilson, 2001a; b). *A. victoriae* was found to be sister to a clade comprising *A. synchronicia*, and in turn to *A. chartacea* and *A. ryaniana*. The relationship of *A. chartacea* and *A. ryaniana* is strongly supported (PP = 1.0). No data exists yet on the salinity tolerance of species closely related to *A. victoriae*. *A. victoriae* is of interests also due to its seed pods being a source of Avicins, which have a strong potential as anti-tumor drugs (Lemeshko, Haridas, Quijano-Pérez, & Gutterman, 2006).

A. salicina also has strong potential in medicinal chemistry, with its leaves having bioactive compounds that have anti-mutagenic, anti-genotoxic and antioxidant potency (Chatti, Boubaker, Skandrani, Bhourri, Ghedira, & Ghedira, 2011; Boubaker, Mansour, Ghedira, & L. Ghedira, 2012). *A. salicina* has been shown to be sister (PP = 1.0) to a clade comprising *A. cupularis*, *A. ligulata*, *A. rostellifera*, *A. xanthina* and *A. tysonii* (Figure 2). *A. ligulata* is described as 'somewhat salt tolerant' based on the salt tolerance of its seedlings (Yokota, 2003). *A. ligulata* is a prospective candidate for revegetation of areas with slight to moderate salinity, *A. xanthina* is recorded to grow on arid lands and limestone, while *A. tysonii* is a species with hard wood, and is advantageous for soil stabilisation in saline sites (World Wide Wattle; <http://www.worldwidewattle.com/>). Thus a number of species in the *A. salicina* clade may be suitable for revegetation purposes.

Ascertaining the relationships of the four target species and their closest relatives based on phylogenetics has helped in narrowing the identification process of putative salt tolerant species. This is significant since there is such limited data on salinity tolerance for the vast number (> 1000) of Australian *Acacia* species, as large scale testing of salinity tolerance parameters is time consuming and prohibitively expensive. As such, a rapid and inexpensive methodology to flag candidate species as described here, is highly significant. The utilisation of DNA markers such as ITS and ETS, is highly informative due to the large amount of already available sequence data. The molecular phylogenetic screening could be followed by testing the select species for physiological markers such as biomass and ion accumulations in laboratory and eventually field conditions. The selected molecular phylogeny approach could be extended to other taxa, or tolerance to other traits such as frost, drought, alkalinity or water logging often associated with salinity tolerance, or phytochemical composition, weediness potential, forage potential, and other characteristics important for utilisation. Thus the results presented here may help in rational selection of candidate plants that not only provide a 'green cover' for the landscape but also contribute to its productivity.

Bui (2013) provide strong evidence that salt stress may have been a crucial element in the adaptation and evolution of Australian vegetation and propose that it may also have been involved in the speciation of acacias. *A. harpophylla*, *A. cambagei* and *A. argyrodendron* belong to the same clade and all can grow on alkaline and saline soils; however, not all species related to these can grow on saline lands. Species that grew on saline as well as alkaline soils were found to form distinct groups within the clade (Bui & Henderson, 2003; Bui, 2013). The reports show that molecular phylogeny can serve as a useful tool for envisaging plant growth characteristics based on edaphic, climatic and biogeographical factors. The method could also provide candidates from unrelated genera, based on species richness of a specific landscape.

5. Conclusion

Many *Acacia* species are capable of withstanding abiotic stress conditions. The preliminary molecular selection process developed here can assist in more rapid selection of native germplasm for field testing, to identify suitable candidates for agroforestry, land reclamation and biodiversity conservation, without the need for transgenic technologies.

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