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Department of Environment and Conservation

FINAL REPORT

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BHP Billiton Yeelirrie Uranium Project

Taxonomic resolution of *Atriplex* sp. Yeelirrie Station (L. Trotter & A. Douglas LCH 25025) utilising morphological and molecular methods

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Summary

Atriplex sp. Yeelirrie Station (L. Trotter & A. Douglas LCH 25025) is supported as a distinct taxon differing from other known species of *Atriplex* based on both morphological and molecular evidence. A molecular study using AFLP markers did not detect a significant genetic difference between two morphotypes (adorned and unadorned fruits) present within *A.* sp. Yeelirrie Station. However, the two known populations of *A.* sp. Yeelirrie Station were found to be genetically remarkably distinct. This is congruent with recent findings in a concurrent population-level analysis using microsatellites by Clarke *et al.* (2011). It is unclear if the difference between the two populations of *A.* sp. Yeelirrie Station supports the recognition of two distinct taxa. The level of differentiation observed is similar to that identified between two subspecies of the related *A. nummularia* (Sampson & Byrne, 2011).

Project Aim

Resolution of the taxonomic status of *Atriplex* sp. Yeelirrie Station (L. Trotter & A. Douglas LCH 25025) and clarification of genetic diversity between morphotypes within this Priority One putative taxon, to inform environmental impact assessment processes.

Project Background

Atriplex sp. Yeelirrie Station (L. Trotter & A. Douglas LCH 25025) is a perennial, dioecious shrub currently known from two populations approximately 30 km apart on Yeelirrie Station, 60 km west of the BHP Billiton Mt Keith Nickel Operation. *Atriplex* sp. Yeelirrie Station occurs along palaeo-channels on self-mulching clays over calcrete. One population occurs near the boundary between Yeelirrie Station and Albion Downs Station (hereafter referred to as "Albion Downs population") and comprises approximately 168,800 individuals. The second population is *c.* 30 km to the north-west on Yeelirrie Station (hereafter referred to as the "Yeelirrie Station population") and comprises approximately 80,600 individuals.

A preliminary assessment of *A.* sp. Yeelirrie Station in 2010 supported it as a taxon distinct from collections of known species of *Atriplex* currently lodged in the Western Australian Herbarium (Shepherd 2010). The preliminary study identified two morphotypes (adorned and unadorned fruiting bracteoles) among the voucher specimens of *A.* sp. Yeelirrie Station available at that time. The preliminary assessment could not address the significance of this variation or determine whether *A.* sp. Yeelirrie Station was one morphologically variable taxon or two taxa.

Consequently BHP Billiton Uranium funded DEC to undertake an integrated morphological and molecular study to:

- determine morphological characters that distinguish *Atriplex* sp. Yeelirrie Station from related species of *Atriplex*;
- confirm the genetic distinctiveness of *Atriplex* sp. Yeelirrie Station and its evolutionary relationships with other species; and
- ascertain the status of the adorned and unadorned fruiting bracteole morphotypes of *A.* sp. Yeelirrie Station using Amplified Fragment Length Polymorphisms (AFLPs).

Morphological assessment of A. sp. Yeelirrie Station

The last formal revision of Australian representatives of the genus *Atriplex* was completed by Paul G. Wilson for the *Flora of Australia* project (Wilson 1984). Wilson (retired) is an active Research Associate at the Western Australian Herbarium. In 2009, following the discovery of an unusual *Atriplex* at Yeelirrie Station, Wilson determined that it represented a potentially new taxon, subsequently phrase-named *Atriplex* sp. Yeelirrie Station (L. Trotter & A. Douglas LCH 25025) (Western Australian Herbarium 1998–). Wilson suggested that the new taxon was possibly allied to the Priority Three species *Atriplex flabelliformis* from the Pilbara region in Western Australia.

Shepherd (2010), in a preliminary assessment of *A.* sp. Yeelirrie Station, noted that two morphotypes with distinctly different fruiting bracteoles were evident within each population. Wilson (1984) had previously noted that many species of *Atriplex* show considerable morphological variation across their range, particularly in the shape and size of leaves and fruiting bracteoles, and that some of this variation may indicate new taxa that had not been recognised due to lack of adequate collections and critical taxonomic assessment. He also noted that taxonomic boundaries between species were not always clear, and that intergrades may occur between allied taxa.

Species of *Atriplex* potentially allied to *A*. sp. Yeelirrie Station occur across central and southern Australia. As part of this morphological study, collections of *Atriplex* held in the State Herbarium of South Australia were examined to augment those held at the Western Australian Herbarium and to assess whether *A*. sp. Yeelirrie Station occurs outside Western Australia. This phase of the study was undertaken prior to results from the molecular sequencing analysis. At this time, *A. flabelliformis* was considered to be the putatively closest allied species to *A*. sp. Yeelirrie Station following the initial assessment of Wilson. The molecular sequencing analysis (see below) indicated that *A*. sp. Yeelirrie Station is more closely allied to *A. cinerea* and *A. amnicola* than to *A. flabelliformis*. The morphological assessment was designed to confirm that morphological characters distinguished *A*. sp. Yeelirrie Station from all potentially related species, both in Western Australia and elsewhere in Australia.

Methods

Atriplex comprises *c*. 62 species in Australia. Many species are dominant in shrubland vegetation in semi arid and arid regions of Australia. Currently there are 2963 collections of *Atriplex* lodged at the Western Australian Herbarium (PERTH) and 7670 collections in the State Herbarium of South Australia (AD). Taxa examined during this study included all potentially allied species as proposed by Wilson, species that had similar morphological features to *A*. sp. Yeelirrie Station, and taxa indicated as related by the molecular phylogenetic results.

Herbarium specimens were examined using a dissecting light microscope with measurements taken using Vernier calipers and a microscope graticule. Floral and fruit material were rehydrated in a weak solution of hot water and detergent for microscopic examination.

Phase 1 of the morphological study

Atriplex sp. Yeelirrie Station

A. sp. Yeelirrie Station is predominantly dioecious, with male and female flowers usually present on separate plants. The leaves are variable in size and shape and the margins may be entire or shallowly toothed. Leaves may be small, rounded and clustered tightly together on the branchlets (Figure 1) or larger and more dispersed (Figure 2).

Figure 1. Small rounded leaves clustered together on a branchlet of *A*. sp. Yeelirrie Station. Voucher specimen KS1377.

Figure 2. On new growth (perhaps following good rainfall) the leaves of *A*. sp. Yeelirrie Station may be larger in size and more scattered along branchlets. Voucher specimen KS1378.

Male flowers are 1.5–1.7 mm long and lack bracteoles. They have a 5-lobed perianth and five stamens. The staminal filaments are 1.0–1.1 mm long and the anthers may change colour from pale yellow to reddish as the age (Figures 3–5).

Figure 3. Male flowers of *A*. sp. Yeelirrie Station generally form dense terminal glomerules at the end of the branchlets. Voucher specimen KS1404.

Figure 4. Male flowers of *A*. sp. Yeelirrie Station comprise a 5-lobed perianth and five pale yellow anthers. Voucher specimen KS1410

Figure 5. As the male flowers of *A*. sp. Yeelirrie Station mature the filaments extend outwards facilitating wind dispersal of the pollen grains. Voucher specimen KS1388

Female flowers lack a perianth and are enclosed between two prominent bracteoles, with only the small stigmas visible at flowering time (Figure 6).

Figure 6. Female flowers of *A*. sp. Yeelirrie Station are difficult to discern except for the emergent stigmas (black arrow). Voucher specimen KS1383.

At fruiting stage, the bracteoles may be unadorned (Morphotype 1; Figure 7) or adorned with globular or finger-like appendages on the outer spongy lobes (Morphotype 2; Figure 8).

Figure 7. Unadorned fruiting bracteoles (morphotype 1) of *A*. sp. Yeelirrie Station. Voucher specimens KS1383 and KS1406.

Figure 8. Adorned fruiting bracteoles (morphotype 2) of *A*. sp. Yeelirrie Station. Voucher specimens KS1411 and KS1409.

Seed morphology is important in species recognition and delimitation in *Atriplex*. Examination of seeds present among the limited specimens available during the preliminary taxonomic assessment (Shepherd 2010) indicated slight differences in seed size between the two morphotypes, with unadorned fruiting bracteoles containing slightly larger seeds. A larger sampling of seeds for this study did not show significant differences between seed size of specimens with unadorned or adorned fruiting bracteoles or between specimens from the Yeelirrie Station or Albion Downs populations (Table 1).

The morphological characters that characterise *A*. sp. Yeelirrie Station are as follows: predominantly dioecious perennial shrubs with divaricate woody branches, with leaves scattered along branches or in clusters; leaf lamina broadly elliptic to ovate, 0.8–6.5 mm long, 0.9–3.2 mm wide, the margin entire or slightly undulating; fruiting bracteoles 2–7 mm long, 1.8–7.4 mm wide, sessile or with a thickened base that expands into fan-shaped lobes fused in the lower half, the valves equal in length, with margins undulate to broadly dentate, with or without appendages and multiple tubercles on the outer face of the valves. Morphological variation in these features is evident within some voucher specimens of *A*. sp. Yeelirrie Station (Figure 9).

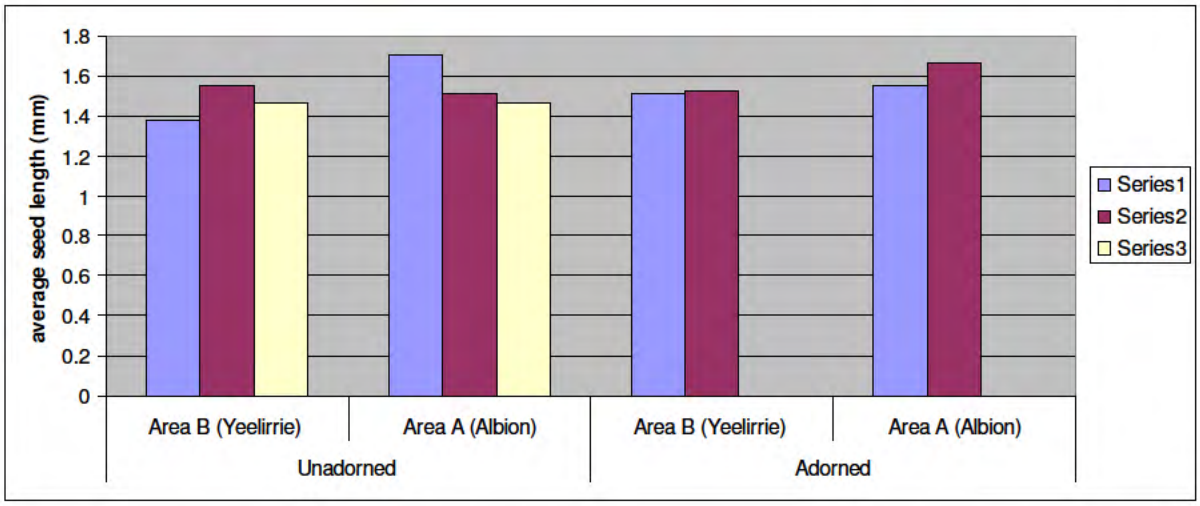

Table 1. Average size of seeds from a limited number of voucher specimens of the unadorned and adorned fruiting bracteole morphotypes from both the Yeelirrie Station and Albion Downs populations.

Figure 9. Leaves and fruiting bracteoles of *A.* sp. Yeelirrie Station. Unadorned morphotype taken from specimen PERTH02373866 (top and middle); adorned morphotype KS1378 (bottom). Scale bars = 3 cm.

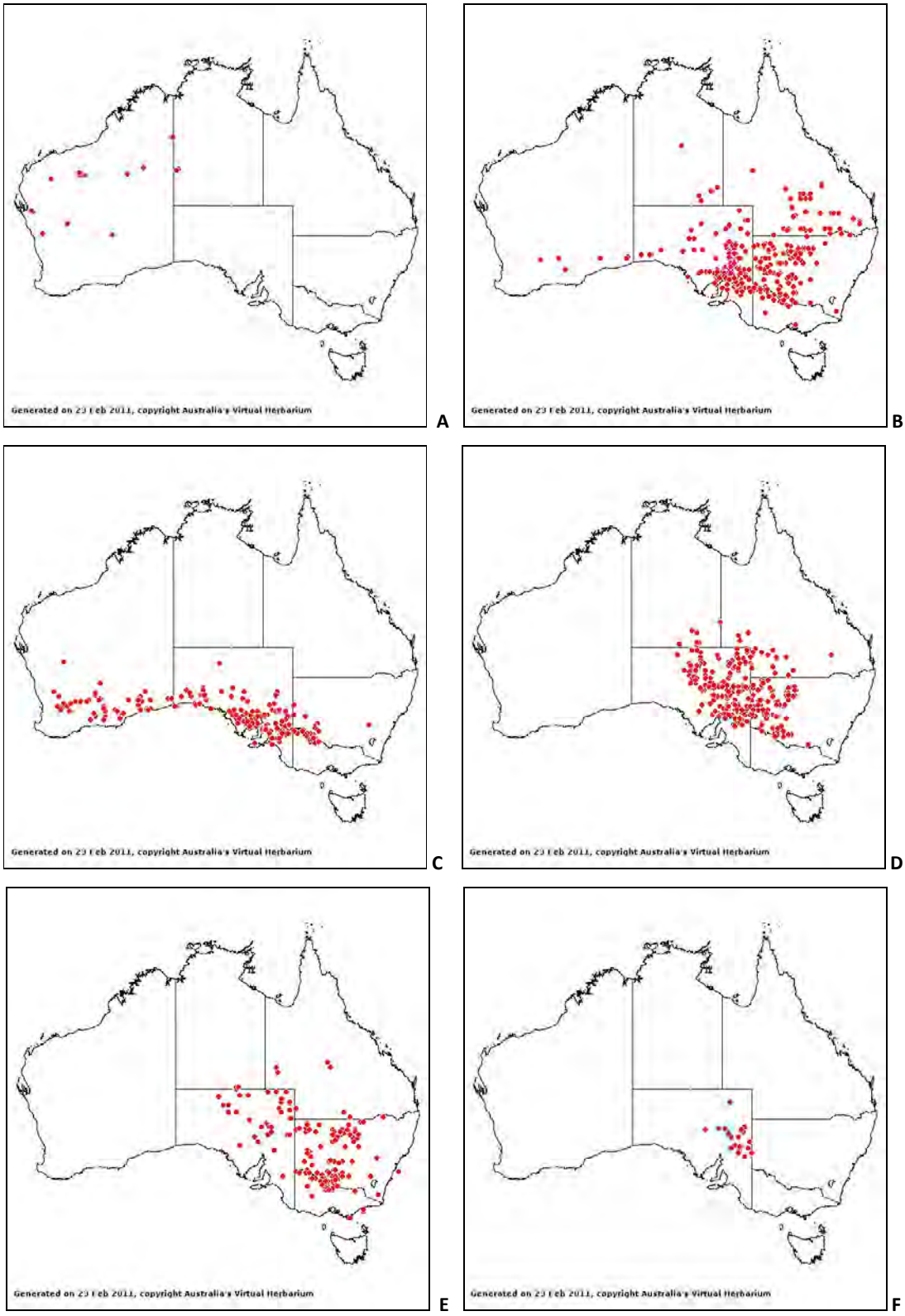

Figure 10. Distribution of widespread species of *Atriplex*, including those not currently known in Western Australia, that were morphologically examined to determine if they were potentially allied to *Atriplex* sp. Yeelirrie Station. A) *A. flabelliformis*, B) *A. eardleyae*, C) *A. acutibractea*, D) *A. angulata*, E) *A. pseudocampanulata,* F) *A. eichleri.*

The morphological features that characterise *A*. sp. Yeelirrie Station were assessed on a number of other potentially related species of *Atriplex*, initially focusing on *A. flabelliformis* and related taxa but including also other taxa with fan-shaped and/or appendaged fruiting bracteoles. Morphological characters were summarised for each species following Wilson (1984).

Atriplex flabelliformis

Monoecious perennial herbs with fine twining branches, with leaves scattered along branches; leaf lamina narrowly elliptic to elliptic, to 10 mm long, the margins entire or slightly sinuate; fruiting bracteoles to 1.5 mm high and 3.5 mm wide, sessile or with a short base that expands into broadly deltoid to fan-shaped lobes fused in the lower half, the valves equal in length, the margins sinuate, with slender papillae on the outer face at the base of the valves near the apex of the short tube (Figure 11).

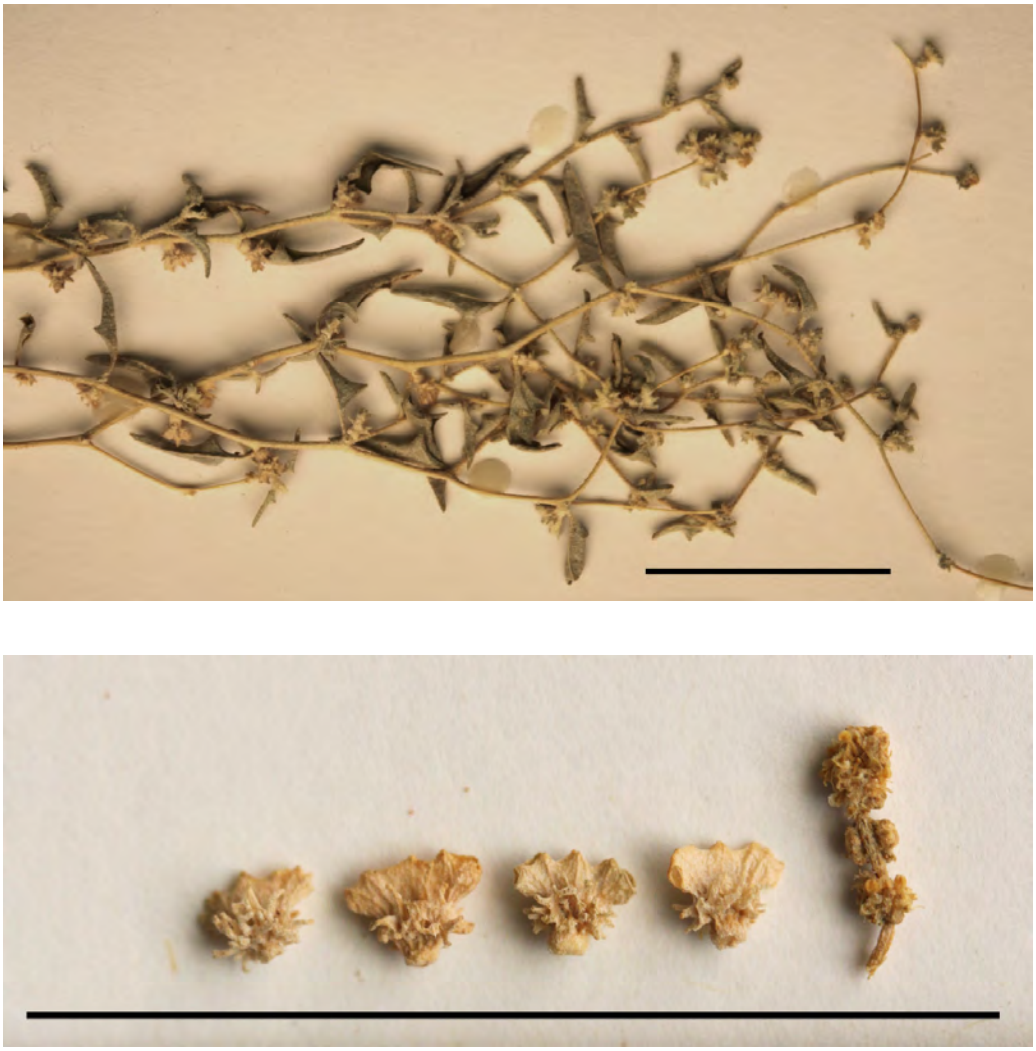

Figure 11. Leaves and fruiting bracteoles of *A. flabelliformis* taken from specimens AD169880980 and AD98037078 respectively. Scale bars = 3 cm.

Atriplex eardleyae

Monoecious perennial herbs with fine twining branches, with leaves scattered along branches; leaf lamina elliptic to orbicular, to 10 mm long, the margins entire; fruiting bracteoles 5.0–7.8 mm long, 9.8–11.5 mm wide, with a narrow elongated campanulate to deltoid tube expanding into broadly deltoid, fan-shaped lobes usually equal in length (sometimes with the adaxial valve shorter than the abaxial valve), the margins sinuate, with a pair of flattened appendages on the adaxial valve near the apex of the tube (Figure 12).

Figure 12. Leaves and fruiting bracteoles of *A. eardleyae* taken from specimen AD97747289. Scale bars = 3 cm.

Atriplex acutibractea

Monoecious perennial shrubs, with leaves in dense clusters or scattered along branches; leaf lamina broadly obovate to orbicular, 5–30 mm long, the margin shallowly sinuate-dentate; fruiting bracteoles 4.2–5.5 mm wide, with a swollen base, with two equal valves fused almost to the apex, the margin with three acuminate to acute points, 2–8 mm long, with small, paired conical appendages usually present at the base of the valves (Figure 13).

Figure 13. Leaves and fruiting bracteoles of *A. acutibractea* taken from specimen AD98922289. Scale bars = 3 cm.

Atriplex angulata

Monoecious annual or short lived perennial herbs with large leaves scattered along branches; leaf lamina broadly obovate to rhomboid with a long petiole, up to 20–40 mm long, the margin entire to sinuate or dentate; fruiting bracteoles 12.7–17.9 mm long, 20.8–25 mm wide, with a narrow, elongated, campanulate to cylindrical tube sometimes thickened around the middle, expanding into free, broadly fan-shaped to deltoid valves usually equal in length, the margins sinuate, without appendages or with two small bumps apparent at the apex of the tube (Figure 14).

Figure 14. Leaves and fruiting bracteoles of *A. angulata* taken from specimen AD98425295. Scale bars = 3 cm.

Atriplex pseudocampanulata

Spreading monoecious herbs with leaves scattered along the branches; leaf lamina elliptic, to 10 mm long, the margin entire or sinuate; fruiting bracteoles 2–3.3 mm long, 3–6.8 mm wide, with a narrow stipe expanding into broadly rhomboid to deltoid, equal valves united in the lower half, sometimes nerved, the margins dentate, often with small tubercles on one surface near the base (Figure 15).

Figure 15. Leaves and fruiting bracteoles of *A. pseudocampanulata* taken from specimen AD98412053. Scale bars = 3 cm.

Atriplex eichleri

Monoecious annual to short lived perennials, with leaves scattered along branches or clustered; leaf lamina elliptic to broadly elliptic, 10–15 mm long, sessile, entire or shallowly sinuate to dentate; fruiting bracteoles 7–8 mm long, 9.8–11.5 mm wide, with a narrow elongated campanulate tube expanding into broadly deltoid to triangular unequal valves with the adaxial valve significantly shorter than the abaxial valve, the valves united in the lower half, distinctly reticulate, the margins entire, with two large appendages attached along the lower margins of the adaxial valve becoming curved inwards (Figure 16).

Figure 16. Leaves and fruiting bracteoles of *A. eichleri* taken from specimen AD99526077. Scale bars = 3 cm.

Phase 2 of the morphological study

A molecular sequencing analysis (see below) indicated that *A*. sp. Yeelirrie Station is closely allied to *A. cinerea, A. amnicola* and related species; accordingly, these species were also assessed morphologically.

Atriplex cinerea

Large dioecious or monoecious perennial shrubs, with leaves shortly petiolate and scattered along branches; leaf lamina narrowly elliptic, elliptic to ovate, 25–40 mm long, the margin entire; fruiting bracteoles 4.5–10 mm long, 4.5–6.9(–10) mm wide, almost sessile or with a narrow tube, expanding into obovoid, broadly deltoid to rhomboid, thickened, equal-sized valves that are free towards the apex and becoming thin, with entire margins, without appendages (Figure 17).

Figure 17. Leaves and fruiting bracteoles of *A. cinerea* from specimen PERTH02375672. Scale bars = 3 cm.

Atriplex amnicola

Large, dioecious, perennial shrubs with leaves that are subsessile to shortly petiolate and scattered along woody divaricate branches; leaf lamina narrowly elliptic, narrowly oblong to hastate, 10–25 mm long, with margins entire or shallowly dentate; fruiting bracteoles 3.6–5.9(–10) mm long, 6.3–14.5 mm wide, almost sessile or with a short, thickened tube expanding into ovoid to deltoid, thickened, equal-sized valves that are fused along the lateral entire margins, without appendages (Figure 18).

Figure 18. Leaves and fruiting bracteoles of *A. amnicola* from specimen AD97346317. Scale bars = 3 cm.

Atriplex rhagodioides is also allied to *A.* sp. Yeelirrie Station but specimens were unavailable for examination at the Western Australian Herbarium. The distribution and taxonomic status of this species is unclear (see below).

It is apparent from this morphological study that *A. amnicola* and *A. cinerea*, with their large leaves and thickened fruiting bracteoles with an acute apex, are more similar to each other than either species is to *A.* sp. Yeelirrie Station. It is unexpected that *A. cinerea*, a widespread coastal species with large leaves, is the most closely allied species to *A.* sp. Yeelirrie Station.

Atriplex eremitis from the Pilbara region may be closely allied to *A. cinerea* and *A. amnicola* (Cranfield, 2008), distinguished from them by its smaller leaves and much smaller fruiting bracteoles reminiscent of those in *A. flabelliformis*. This species is also distinct from *A.* sp. Yeelirrie Station (Table 2).

		A. eremitis	***A. cinerea***	***A. amnicola***	***A.* sp. Yeelirrie Station**
Leaf	Outline	entire	entire	entire-hastate to shallowly dentate	entire to shallowly sinuate
	Length (mm)	4–16	25–40	10–25	0.8–6.5
	Lamina shape	elliptic-oblong	elliptic-ovate	elliptic-oblong to hastate	broadly elliptic to ovate or hastate
	Lamina base	attenuate	rounded	cuneate-hastate	rounded
Fruiting bracteole	Attachment	sessile	stipitate-subsessile	sessile	Sessile or thickened base
	Length (mm)	1.5–2	4.5–10	(3.6–)6–10	2–7
	Width (mm)	1.5–2	4.5–10	4–6(–14.5)	1.8–7.4

Table 2. Leaf and fruiting bracteole characters of *A. eremitis*, *A. cinerea*, *A. amnicola* and *A.* sp. Yeelirrie Station as modified from Cranfield 2008.

In summary, *Atriplex* sp. Yeelirrie Station is morphologically distinct from all known taxa, particularly from those that on molecular evidence are its closest relatives.

Molecular assessment of A. sp. Yeelirrie Station relative to other species of Atriplex

Molecular sequencing of plant DNA is a powerful tool when used in conjunction with traditional morphological studies to elucidate evolutionary relationships among related species and higher taxonomic groups. One of the most commonly sequenced regions used in studies to infer relationships between closely related species is the Internal Transcribed Spacer (ITS) region of the nuclear 18S–26S ribosomal DNA (nrDNA) gene. The ITS region often shows significant variation between related species, commonly attributed to the fact that it is non-coding; mutations in this region are not exposed to selection and hence persist through time. Thus, degrees of relatedness between species may be inferred by assessing shared mutations in the ITS sequence. However, ITS is not always able to distinguish closely related species (Klak *et al*., 2003; Ladiges *et al*., 2003; Murphy *et al*., 2003; Shepherd *et al.* 2004) if the taxa have recently speciated or if hybridisation has occurred between related species. In recent years it has become common to sequence, in addition to ITS, the related External Spacer Region (ETS), which in some cases has been found to evolve faster than ITS and hence may be more informative (Benna *et al.* 1998).

Kadereit *et al.* (2010) sequenced ITS and ETS from over 200 species of *Atriplex* from throughout the world, including 62 species from Australia, to construct a detailed molecular phylogeny for the genus. Sequences of these regions in *Atriplex* sp. Yeelirrie Station for this study has provided an independent means of confirming if it is genetically distinct, and of placing it within a broader evolutionary framework.

Methods

Fresh leaf material of *Atriplex* sp. Yeelirrie Station (*K.A. Shepherd, B. Graham & C. Ringrose* KS 1381) from Yeelirrie Station was dried in silica gel and supplied to Dr Gudrun Kadereit at the Johannes Gutenberg-Universität in Germany for ITS and ETS sequencing.

Methods and analysis follows Kadereit *et al.* (2010). The ITS and ETS sequence data were analysed separately and then as a combined data set using BEAST (Bayesian Evolutionary Analysis by Sampling Trees v1.4.8), which estimates tree topology and divergence times.

Results and Discussion

There were more than 10 base pair differences between *Atriplex* sp. Yeelirrie Station and the closest other species of *Atriplex* (S. Krieg *pers. comm.*). Analyses of the ITS and ETS sequence data sets were congruent, and therefore could be combined. The resulting phylogeny (Figure 19) supported *A.* sp. Yeelirrie Station as distinct, and most closely allied to the widespread and predominantly coastal species *A. cinerea*, which is found across southern Australia extending to Lord Howe Island and New Zealand (Figure 20).

These taxa are sister to a clade including *A. rhagodioides* and *A. amnicola. A. rhagodioides* was originally thought to be confined to the Murray River region but specimens currently housed in the National Herbarium of New South Wales (NSW), Queensland Herbarium (BRI) and the National Herbarium of Victoria (MEL) indicate that this species extends to Western Australia central South Australia and north-western New South Wales (Figure 21). It is likely, however, that these records result from misidentifications in these herbaria and are not *A. rhagodioides*; this needs to be confirmed.

Atriplex amnicola is found along the north-west coast of Western Australia around Shark Bay and inland through the arid zone (Figure 22). Other allied species include the Western Australian coastal species *A. isatidea* (Figure 22) and *A. nummularia*, one of the most variable and widespread Australian species of *Atriplex*. Currently two subspecies are recognised within *A. nummularia*: subsp. *nummularia* found throughout NT, SA, Qld, NSW and Vic and subsp. *spathulata* currently known only from WA and SA.

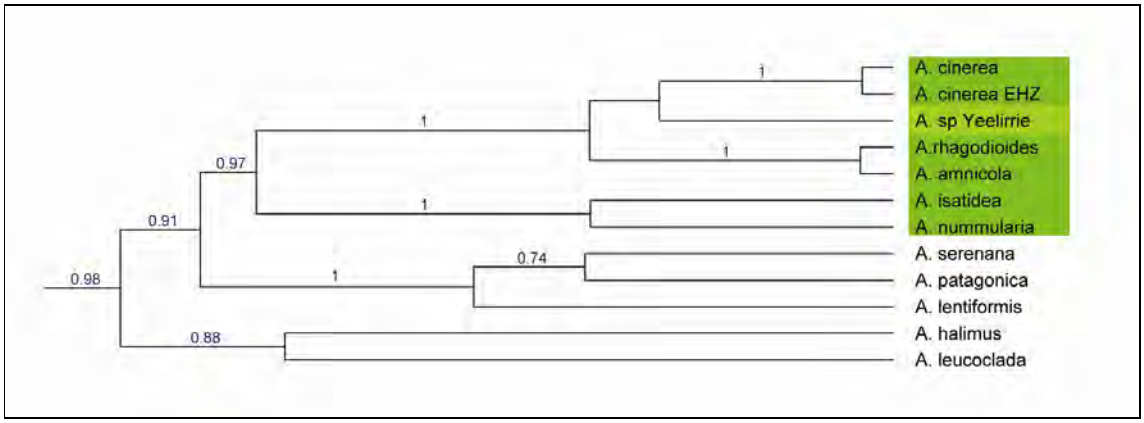

Figure 19. Phylogenetic position of *Atriplex* sp. Yeelirrie Station among related species of *Atriplex* using combined ITS and ETS nrDNA sequences with significant Maximum Likelihood values above supported branches (unpublished data from Krieg & Kadereit).

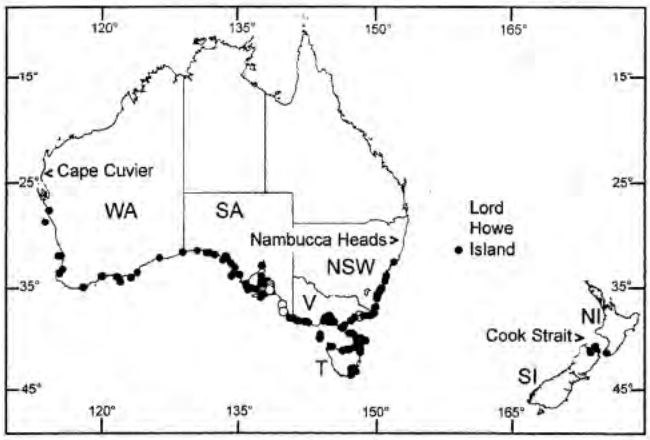

Figure 20. Herbarium collections and field observations of *A. cinerea* included in the study by P.C. Heyligers (2001).

The phylogenetic clade comprising *A.* sp. Yeelirrie Station and allied species corresponds to a group recovered in a recent phylogenetic analysis of the tribe Atripliceae within the subfamily Chenopodioideae referred to by Kadereit *et al.* (2010) as 'Australian Clade 1' (Figure 23). The species within this clade are polyploids (they have multiple sets of chromosomes). Findings in a concurrent BHP Uranium funded microsatellite study (Clarke *et al.* in prep.) suggests that *A.* sp. Yeelirrie is hexaploid but the origin and type of polyploidy (allo- or auto-polyploid) is unknown.

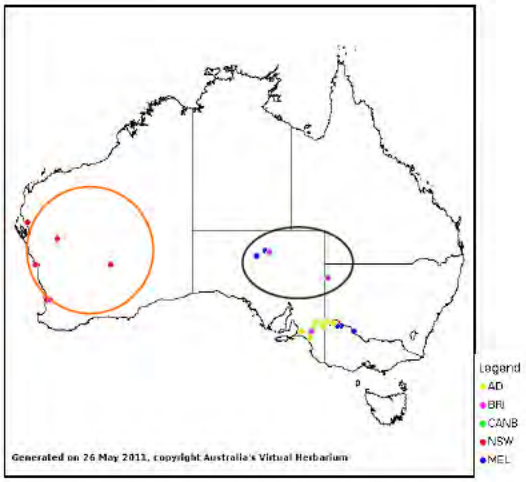

Figure 21. Australia's Virtual Herbarium (AVH http://avh.rbg.vic.gov.au/avh accessed 26 May 2011) records of *Atriplex rhagodioides*. The identification of specimens in Western Australia, central South Australia and north-west New South Wales (circled) as *A. rhagodioides* may be in error, resulting from incorrect determinations on specimens in some herbaria.

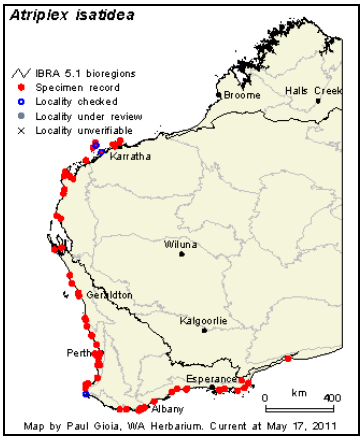

Figure 22. Distribution of Western Australian species *A. amnicola* and *A. isatidea* from FloraBase (http://florabase.dec.wa.gov.au accessed 25 May 2011).

The polyploid Australian species of *Atriplex* in 'Australian Clade 1' are closely related to a very widespread group including *A. serenana* and *A. lentiformis* from southwestern United States, *A. patagonica* from Argentina, *A. halimus* from Europe and Northern Africa and *A. leucoclada*, which originated in the Middle East (Figure 19). This clade is basal to a larger clade that includes other global species of *Atriplex* and a radiation of Australian species referred to by Kadereit *et al.* (2010) as 'Australian Clade 2' (Figure 23). *Atriplex flabelliformis,* originally believed to be related to *A.* sp. Yeelirrie Station on the basis of its fan-like, ornamented fruiting bracteoles is phylogenetically not closely related to *A.* sp. Yeelirrie Station (Figure 23).

Molecular status of the two morphotypes within A. sp Yeelirrie Station

Two morphologically different forms (morphotypes) with adorned or unadorned fruiting bracteoles were observed in the field within populations of *A.* sp Yeelirrie Station. This portion of the study aimed to determine if this morphological variation is associated with significant genetic differences.

Methods

Collections of leaf material were made from adorned and unadorned morphotypes from adjacent plants in the two known populations (Yeelirrie adorned *n* = 16, Yeelirrie unadorned *n* = 22, Albion Downs adorned *n* = 20, Albion Downs unadorned *n* = 19). In addition, samples were collected from seven male plants at the Albion Downs population, five plants at a rehabilitation site on Yeelirrie Station and five male plants from the Yeelirrie Station population to the north-west. AFLP profiles were obtained for these additional samples for comparison, but the data was not included in final analyses.

Genomic DNA was extracted from 50 mg of freeze dried leaf material using a cetyltrimethylammonium bromide (CTAB) method, modified from Doyle and Doyle (1987), with 1% w/v polyvinylpyrrolidone (MW 40,000) added to the extraction buffer.

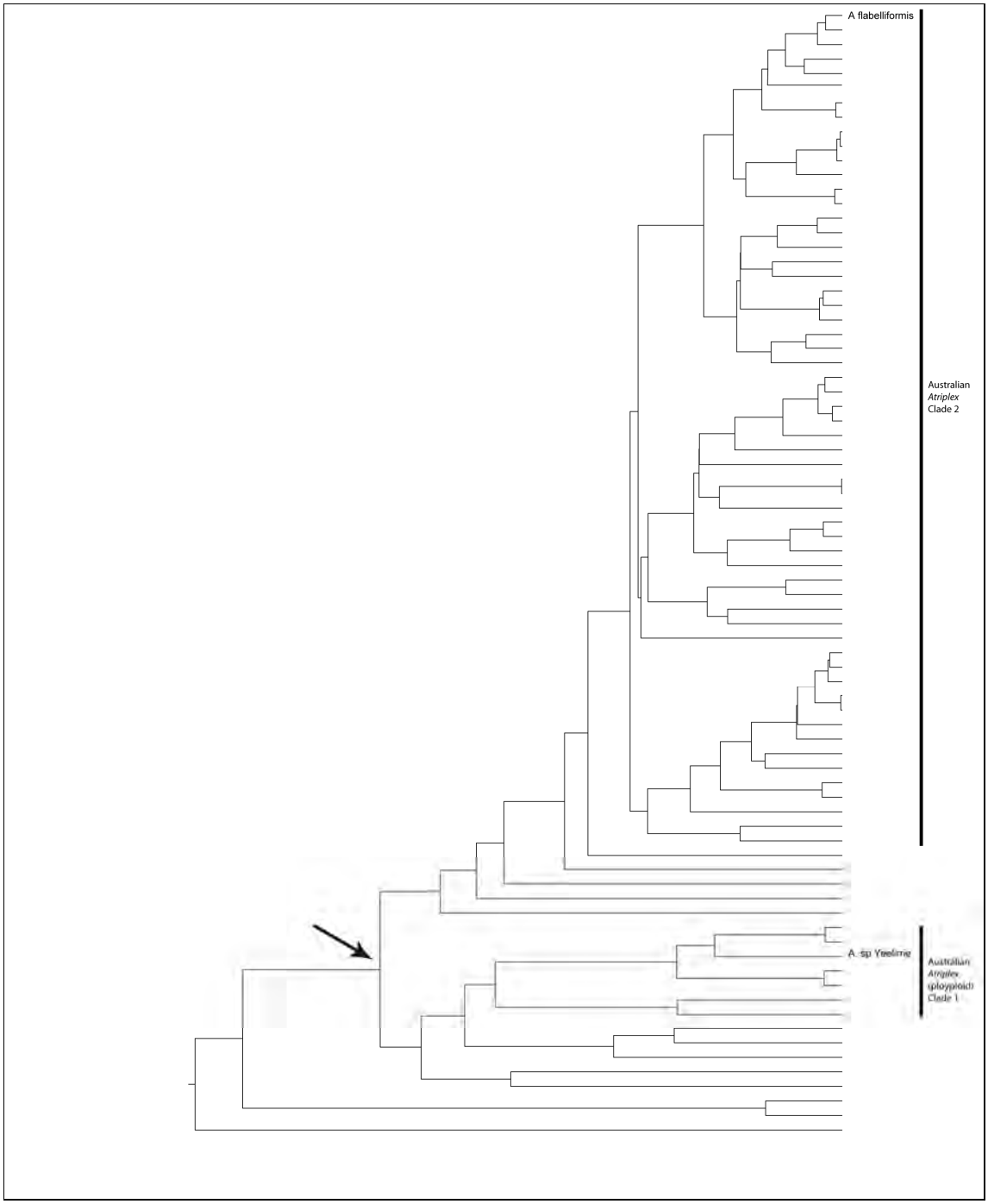

Figure 23. A simplified summary of the broader *Atriplex* phylogeny based on combined ITS and ETS nrDNA sequences, highlighting the position of *A.* sp. Yeelirrie Station within the small polyploid Australian '*Atriplex* Clade 1' and the point of divergence from the larger *Atriplex* group which includes *A. flabelliformis* within the large Australian '*Atriplex* Clade 2' (unpublished data from Krieg & Kadereit).

The Amplified Fragment Length Polymorphism (AFLP) analysis followed the protocol provided by Invitrogen (Life Technologies™). An initial screening of 24 selective primer pair combinations was done using four individuals. Four primer pairs that gave clear, reproducible and polymorphic AFLP profiles were selected: *Eco*RI-AGC-(6-FAM)/*Mse*I-CTC, *Eco*RI-AAC-(VIC)/*Mse*I-CTA, *Eco*RI-ACC-(NED)/*Mse*I-CAA and *Eco*RI-ACG-(PET)/*Mse*I-CTC. Amplification products were separated on an Applied Biosystems 3730 capillary sequencer at the Western Australian State Agricultural Biotechnology Centre, Murdoch University. Size calibration was obtained using an internal size standard GeneScan-500 LIZ® (Applied Biosystems).

AFLP band profiles were scored from the raw data using GENEMAPPER version 4.0. The partitioning of genetic diversity within and among populations and morphotypes was estimated by the analysis of molecular variation method (AMOVA) in GenAlEx 6.4 (Peakall and Smouse 2006). AMOVA was conducted with individuals grouped into a hierarchy of species, populations and morphotypes. Relationships between individuals were also described by a Principal Components Analysis (PCA) of a matrix of pairwise genetic distances between individuals calculated using GenAlEx v6 (Peakall and Smouse, 2006). A Bayesian analysis using the program STRUCTURE 2.3.3 (Pritchard, Stephens & Donnelly, 2000) and the approach developed by Falush, Stephens and Pritchard (2003, 2007) was undertaken to identify clusters of individuals on the basis of multilocus genetic data.

Results

847 polymorphic AFLP fragments were detected within the range 50–500 base pairs (bp). Each individual possessed a unique AFLP fingerprint.

The PCA, AMOVA and Bayesian analyses all showed no evidence of genetic differentiation associated with morphotype, but strongly supported genetic distinction of the two populations. Analysis of variation using AMOVA showed that 85% of genetic diversity was within populations with 15% between populations. When the analysis included a grouping of morphotypes within populations, the pattern remained the same; 85% of diversity was within populations, 15% between populations and less than 0.5% (effectively 0%) between morphotypes within populations.

Grouping of individuals based on a Principal Coordinate Analysis of genetic distance showed morphotypes clustered with individuals from their population of origin, not with similar morphotypes elsewhere (Figure 24). The analysis showed clear separation of the two populations.

The Bayesian analysis clearly identified two clusters corresponding to the two populations with a high average similarity between 20 replicate analyses (H' = 0.998) (Figure 25). The proportion of an individual's genome coming from its population of origin's cluster (q) was high for both populations (Albion Downs 98% ± 5% and Yeelirrie Station 99% ± 3%) and very few individuals showed admixture of the two clusters. There was no clustering associated with morphotype.

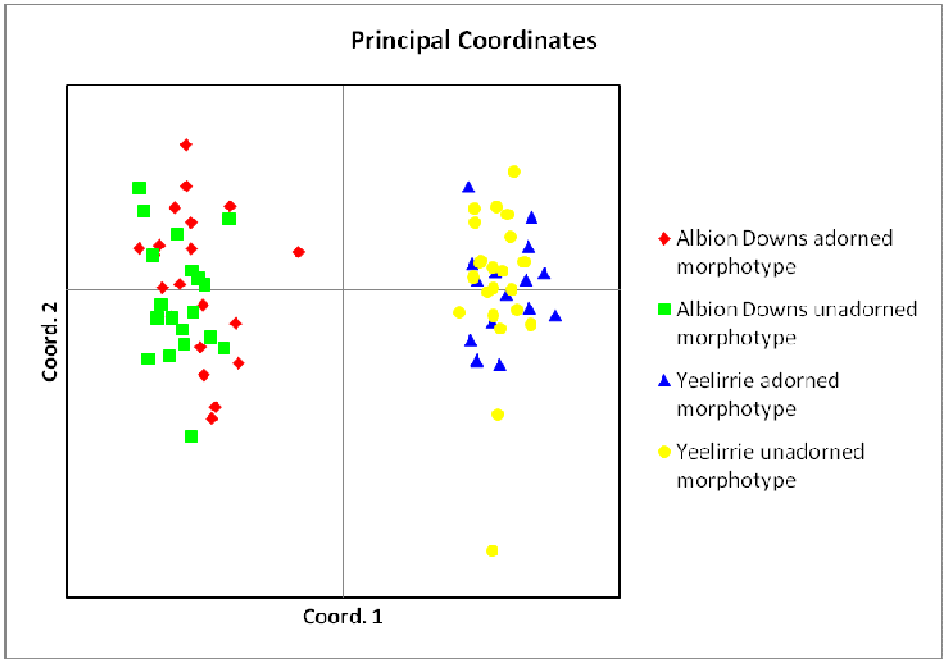

Figure 24. Principal coordinates analysis of pairwise genetic distances between individuals of *Atriplex* sp. Yeelirrie Station based on binary genetic distance.

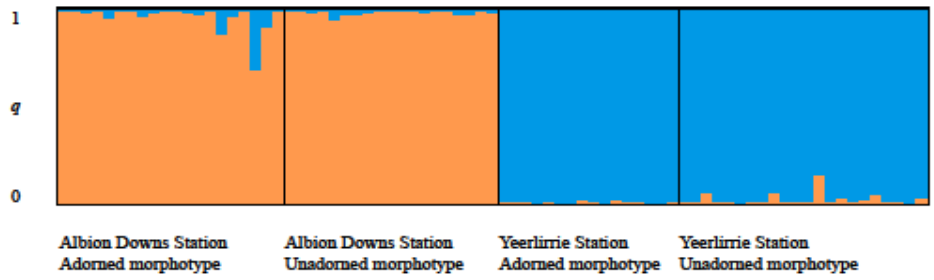

Figure 25. Genetic ancestry of individuals of *Atriplex sp. Yeelirrie* from two populations and two morphotypes estimated using Bayesian analyses and STRUCTURE 2.3.3. Each individual's genome is shown as a bar divided into shaded segments in proportion to the estimated ancestry (*q*) in each cluster. Results shown are the optimal alignment of 20 replicates.

Discussion

The high number of AFLP fragments found in samples of *A.* sp. Yeelirrie from only two populations is consistent with the species being polyploid, as is common among Australian *Atriplex* (Nobs 1980; Parr-Smith 1982). The distribution of diversity within and among populations and morphotypes showed a clear differentiation of populations but there was no differentiation associated with morphotype. The proportion of diversity between populations separated by *c.* 30 km, estimated by AMOVA (15%), was similar to that found between subspecies of the octoploid *Atriplex nummularia* Lindl. using microsatellites (16%, Sampson & Byrne 2011), and higher than that found between populations of *A. nummularia* over it's continent-wide distribution in Australia (11%). This may indicate low gene flow between populations or multiple origins or both, but further sampling and investigation is required to address these questions. In all analyses there

was no genetic structure associated with the adorned or unadorned fruiting bracteole morphotypes.

Genetic diversity and the partitioning of this diversity within and between populations is influenced by a range of factors including population size and distribution as well as the pollination mechanism and mating system of a species. Endangered species with low population numbers have a small gene pool and may undergo genetic erosion and a slow decline in biological fitness. Thus, the genetic diversity of a species will influence its long term survivability and ability to withstand environmental and anthropogenic impacts. *Atriplex*, like other members of the family Chenopodiaceae, have small, wind-pollinated flowers – a feature that promotes outcrossing and in turn would be expected to result in high levels of genetic diversity within species. *A.* sp. Yeelirrie Station is also expected to be strongly outcrossing as it is dioecious; however, the presence of some fruits on male plants (Figure 26) shows that it is partially monoecious, indicating that self-fertilisation may sometimes occur.

Many chenopods produce papery fruits and seeds that may be dispersed long distances by wind or water. In contrast, the fruits of *A.* sp. Yeelirrie Station appear to be retained for long periods, possibly a year or more, as old grey fruits occur on the lower branches while new fruits are maturing near the terminal branches. This may limit the distance seeds are dispersed from maternal plants. *A.* sp. Yeelirrie Station appears also to be restricted to a specific and rare habitat in palaeochannels, reducing the likelihood of plants establishing between populations and acting as a bridge for gene flow. It is unclear if the palaeodrainage channels occupied by the two populations of *A.* sp. Yeelirrie Station become interconnected during flood events. The genetic results suggest that the two populations have been separated for a long period, resulting in a significant level of genetic divergence.

Figure 26. Old grey fruits retained on the lower branches of a male-flowering plant of *Atriplex* sp. Yeelirrie Station. Voucher: K.A. Shepherd KS 1385.

Conclusion and Recommendations

Atriplex sp. Yeelirrie Station is clearly a distinct, new taxon, different from all other known species of *Atriplex* in Australia, and very distinct from its phylogenetically closest relatives.

The two populations of *A.* sp. Yeelirrie Station are genetically very distinct, despite their close proximity. It is critically important to assess whether the two populations are best regarded as separate taxa (species, subspecies or varieties) or as belonging to a single taxon. Two approaches may be taken to allow such an assessment.

The first approach would compare the observed genetic differentiation between the populations with that found in related species, to indicate whether the divergence observed within *A.* sp. Yeelirrie Station is unusually high. Few comparable studies in *Atriplex* are available for such an assessment. An expanded AFLP study should be undertaken to compare the degree of genetic differentiation among and between populations of the closest relatives of *Atriplex* sp. Yeelirrie Station (see Shepherd & Byrne 2011).

The second approach would assess morphological variation between the two populations, to assess whether morphological features can be found that support recognition of the populations as distinct taxa. While no clear morphological separation has been observed between the populations to date, a detailed morphometric analysis should be undertaken for this purpose.

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