

DNA analysis identifies *Solanum* from Litchfield National Park as a lineage of *S. dioicum*

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Abstract

The uncommon reproductive system of dioecy is somewhat widespread in Solanaceae, being exhibited by members of five genera within this family. These members represent, however, only around 1% of species within the Solanaceae. The highest incidence of dioecy is found in the genus *Solanum*, where around 15 species have been described as consisting of populations in which individual plants are either “male” (staminate) or “female” (pistillate). Ten of these *Solanum* species, commonly known as ‘bush tomatoes’, are endemic to the Australian monsoon tropics. During recent fieldwork in the Northern Territory, non-reproductive collections were made of a morphologically distinct population of *Solanum* (*Solanum* sp. Litchfield I.D.Cowie 1428) from Litchfield National Park. We generated the first DNA sequences of these exceptional plants, amplifying the ITS (nuclear) and *trnK-matK* (chloroplast) DNA regions. Phylogenetic analysis comparing molecular data of *Solanum* sp. Litchfield with previously sequenced relatives infers that the taxon is closely allied to *S. dioicum*, a widespread species already considered to be morphologically diverse. However, we consider *Solanum* sp. Litchfield to represent a morphologically and geographically distinct taxon. Although all specimens collected to date lack reproductive features, the phylogenetic placement of *Solanum* sp. Litchfield infers that the species is likely to be dioecious, thus broadening our understanding of the distribution and circumscription of dioecious lineages of *Solanum* in Australia.

Introduction

Within the plant family Solanaceae, the highest abundance of dioecy (‘male’ and ‘female’ flowers occurring on separate plants) is recorded in the genus *Solanum*, with

around 15 species using this form of reproduction (Anderson & Symon 1989; Martine *et al.* 2006; Martine *et al.* 2009). Most (12) of the known (*c.* 16) dioecious solanums are included in subgenus *Leptostemonum*, which is known as the ‘spiny solanums’ (Miller & Diggle 2003; Martine *et al.* 2009). Ten of these species are found only in northern Australia (Anderson & Symon 1988; Anderson & Symon 1989), where they are known locally as ‘bush tomatoes’. The geographic ranges of these ten species are more or less restricted to two regions: the Kimberley of Western Australia and the central Arnhem Land Plateau of the Northern Territory (Symon 1980; Martine & Anderson 2007; Martine *et al.* 2009).

All occurrences of dioecy in *Solanum* are exhibited through a ‘cryptic’ form in which populations within a species appear to be androdioecious (having male flowers on some plants and hermaphrodite flowers on others). However, these species have been shown to be functionally dioecious because the pollen produced by morphologically hermaphrodite flowers is inaperturate (i.e. without pores) and incapable of germination, making them unable to contribute to male reproductive function through fertilization (Anderson & Symon 1989; Martine *et al.* 2009; Martine *et al.* 2010). Thus, individual dioecious bush tomatoes are either ‘male’ (with clearly staminate flowers bearing only stamens) or ‘cryptically female’ (with conspicuous pistils and stamens producing non-functional pollen).

Because of the frequency of dioecy in Australian *Solanum*, and its occurrence over a restricted geographic region, the bush tomato group has been treated as a model system in which to study the evolution of dioecy, especially because potential transitional reproductive states are also present in the lineage (Anderson & Symon 1989). Current work in *Solanum* subgenus *Leptostemonum* has also focused on the apparent recent radiation of Old World ‘spiny solanums’ (Levin *et al.* 2006; Bohs *et al.* 2007) and it is clear that the evolutionary relationships among most Australian, Asian, and African species of *Solanum* subgenus *Leptostemonum* remain difficult to elucidate without a comprehensive sampling of taxa and the use of more informative gene regions than currently used (Bohs *et al.* 2007).

Although recent work by Martine and colleagues (Brennan *et al.* 2006; Martine *et al.* 2006; Martine & Anderson 2007; Martine *et al.* 2009) has built on the outstanding contribution of Symon (1980), the taxonomy and circumscription of dioecious Australian solanums is still in flux. Notably, several distinct populations of dioecious solanums with uncertain taxonomic affinity are known and/or have been collected from the Kimberley, the central Arnhem Plateau and adjacent areas (D. Symon, pers. comm). This includes a population from Litchfield National Park that has long been suspected by botanists at the Northern Territory Herbarium (DNA) to be a distinct taxon based on its slender leaves, limited armature and diminutive size (Figure 1). This population has been designated as *Solanum* sp. Litchfield (I.D.Cowie 1428) (Short *et al.* 2011). Collections of non-reproductive material of *Solanum* sp. Litchfield have been

made by K.G. Brennan, I.D. Cowie, J.L. Egan, J.O. Westaway and others and accessioned at the Northern Territory Herbarium.

In the absence of specimens with flowers and fruit, this study uses DNA sequence data to evaluate the phylogenetic position of *Solanum* sp. Litchfield and to assess whether it corresponds to a described taxon or represents a previously undescribed taxon.



Figure 1. Adult non-reproductive specimen of *Solanum* sp. Litchfield I.D.Cowie 1428 on sandstone rockpile along east side of Florence Falls Rd., Litchfield National Park. (F.D. Vogt)

Methods

Understanding the phylogenetic placement of *Solanum* sp. Litchfield requires two steps: (1) obtaining DNA sequence data, and (2) appending the new DNA sequences to available data from related species (Table 1). The two DNA regions selected for this study were the ITS (nuclear) and *trnK-matK* (chloroplast) regions, which Martine *et al.* (2009) used to estimate phylogenetic relationships among about 25 closely-related species of *Solanum* endemic to Australia.

Table 1. Previously sequenced Australian dioecious species of *Solanum* (Martine *et al.* 2009) used for comparison with new sequences generated for *Solanum* sp. Litchfield, including an undescribed species from the Kimberley coast (*S.* sp. 'Longini'). The dataset consists of nine andromonoecious species from Australia included by Martine *et al.* (2009) in previous phylogenetic studies.

Dioecious species	Center of distribution	GenBank
<i>S. asymmetriphyllum</i>	Kakadu	EU983570
<i>S. carduiforme</i>	Kimberley/NT	EU983556
<i>S. dioicum</i> (typical)	Kimberley	EU983553
<i>S. dioicum</i> (Tanami Desert form)	Kimberley	EU983554
<i>S. leopoldensis</i>	Kimberley	EU983560
<i>S. petraeum</i>	Kimberley	EU983559
<i>S. sejunctum</i>	Kakadu	EU983568
<i>S. tudununggae</i>	Kimberley	EU983552
<i>S.</i> sp. 'Longini'	Kimberley	EU983561

Collections

Field collections (Table 2) were made in May 2009 in Litchfield National Park, in localities suggested by staff botanists in the Northern Territory Herbarium. Voucher specimens were pressed for herbarium accessioning, while fresh leaf samples were placed in individually labeled envelopes partly filled with silica gel (to quickly preserve leaves to be used for later DNA extractions).

DNA isolation and PCR amplifications:

DNA was isolated from silica-dried leaf material using a modified standard protocol (Doyle & Doyle 1987). Each gene region was PCR-amplified using primers for the ITS region and *trnK-matK* region (Table 3) using the protocols described by Martine *et al.* (2006, 2009). Because of the large size of the *trnK-matK* target region, primers were used that would create overlapping fragments to be assembled into one large contiguous sequence. Presence of PCR product was confirmed on 1% agarose gel.

Table 2. Collections of *Solanum* sp. Litchfield made during the 2009 expedition. All collections were made from within Litchfield National Park.

Collection no.	Locality information	DNA code	GenBank accession #	
			ITS	<i>trnK-matK</i>
CTM 1751	Sandstone rockpiles along east side of Florence Falls Rd, 0.5 km N of junction with Litchfield Park Rd. 13°07.531'S, 130°48.140'E	01A	JN098472	JN098476
CTM 1752	Near above	01B	JN098473	JN098477
CTM 1753	Sandstone rockpiles in vicinity of 'The Lost City,' 150-200 m SE of car park. 13°13.137'S, 130°44.216'E	04A	JN098474	JN098478
CTM 1754	Near above	04B	JN098475	JN098479
CTM 1756	Sandstone rockpiles just south of junction of Litchfield Park Rd. and Florence Falls Rd. 13°07.664'S, 130°48.305'E	N/A		

Table 3. Gene regions examined and associated primer sequences.

Gene Region	Primer Sequence
<i>ITS-4</i>	TCCTCCGCTTATTGATATGC
<i>ITS-5</i>	GGAAGTAAAAGTCGTAACAAGG
<i>68F</i>	TCTTTCAGGAGTATATTTATG
<i>1556R</i>	CCTTGATACCTAACATAATGC

Cycle sequencing

Each PCR reaction was cleaned by adding 0.5 µL of a 1:5 dilution of ExoSAP-IT® (Affymetrix, Inc., Santa Clara, California, USA) to 1.0 µL of PCR product. Cycle sequencing was performed with the same sets of forward and reverse primers used in the PCR reactions using the method described by Martine *et al.* (2006). Raw DNA sequences were then recorded using an ABI Prism® 3100 automated sequencer (Applied Biosystems, Foster City, California, USA).

Sequence alignment and phylogenetic analysis

Editing of raw sequences was performed using 4Peaks software (Griekspoor & Groothuis 2005) for ITS and CodonCode Aligner, version 1.3.1 (CodonCode Corporation, Dedham, Massachusetts, USA) for *trnK-matK*. The two gene regions

were combined to form a dataset of more than 2600 base pairs. A manual alignment was performed in MacClade (Maddison & Maddison 2005) and then exported into PAUP* (Swofford 2002) for phylogenetic analyses using Maximum Parsimony. Bootstrapping was performed with 500 replicates.

Results

The four accessions of *Solanum* sp. Litchfield included in the analysis form a well-supported (93% bootstrap) clade nested within the 'Kimberley Dioecious' clade (Martine *et al.* 2006), a group of several sub-arid and monsoonal dioecious spiny solanums closely allied with *S. dioicum* that occur throughout the Kimberley and sandstone country of the NT. The distinctness of *Solanum* sp. Litchfield was apparent even when each gene region was analyzed separately, but we have chosen to present the relationships based on the combined dataset (Figure 2) so as to remain consistent with the expanded treatment of Martine *et al.* (2009). Although the small range of *Solanum* sp. Litchfield is geographically close to Kakadu National Park, the taxon does not appear to be closely related to *S. sejunctum* and *S. asymmetriphyllum*, the two members of the dioecious 'Kakadu clade' as defined by Martine *et al.* (2006).

Discussion

Phylogenetic analysis using the ITS and *trnK-matK* gene regions supports the distinctness of the *Solanum* population from Litchfield National Park (Figure 2). This conclusion is based on the fact that the DNA sequences extracted from all four collections of *Solanum* sp. Litchfield are shown to be more similar to one another than any of them are to the related species of *Solanum* included in the analysis. The phylogeny presented in Figure 2 clearly illustrates this, with moderate level of support provided by bootstrap analysis. Our analysis using these two DNA regions, one nuclear and one chloroplast, provides further evidence that *Solanum* sp. Litchfield is closely allied to *S. dioicum* and other taxa from the Kimberley region.

Although additional evidence may eventually validate *Solanum* sp. Litchfield to be a distinct species, the current lack of data regarding its reproductive structures allows only for its provisional recognition as a lineage within *S. dioicum*. Prior to our study, *S. dioicum* already was considered to include at least two divergent lineages, one of which (*Solanum dioicum* Tanami Desert form) is known from the eastern Kimberley and is distinct in having broad, densely white-tomentous leaves and heavily armed stems and calyces. The taxonomic disposition of lineages within *S. dioicum* has been difficult to resolve because populations appear to intergrade morphologically (Symon 1980) and because sampling for recent molecular systematic studies has not been broad enough to capture the extent of variation within the complex. A complete taxonomic treatment of *S. dioicum* (including *Solanum* sp. Litchfield) must address the disposition of *Solanum dioicum* Tanami Desert form and other unresolved populations in order to maintain a cladistically consistent *S. dioicum*.

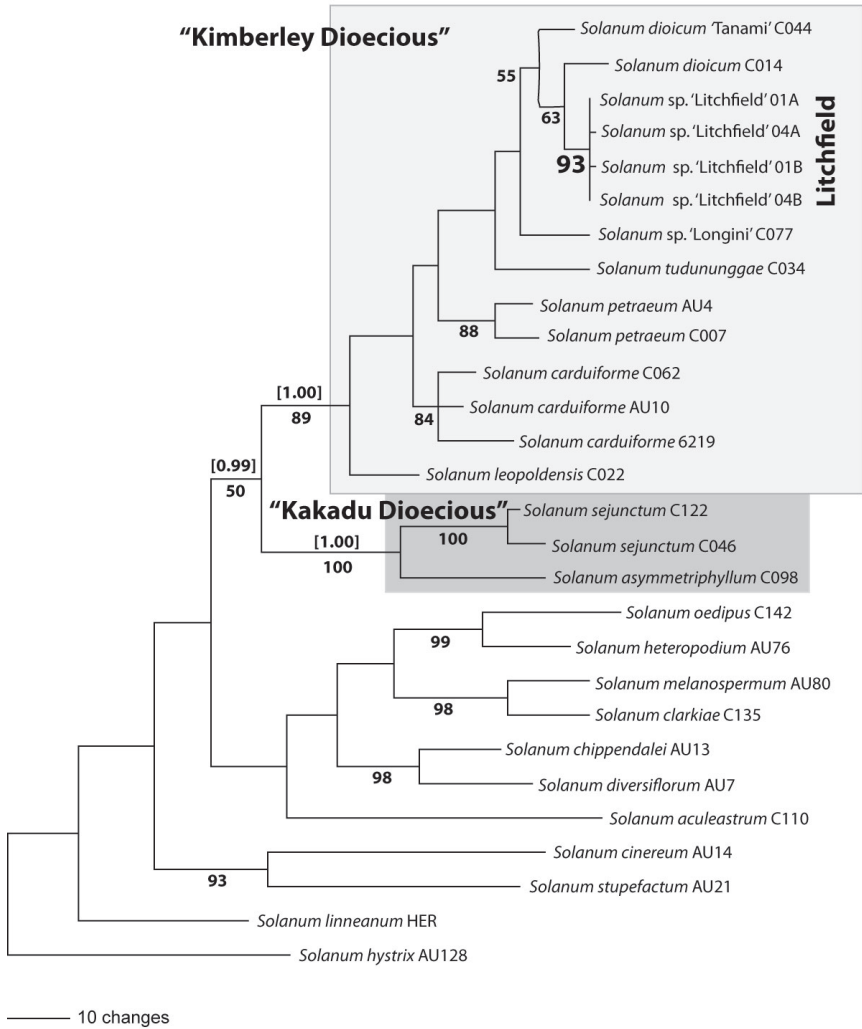


Figure 2. One of a set of best Maximum Parsimony trees depicting the relationship of *Solanum* sp. Litchfield (four accessions) to other dioecious and andromonoecious Australian spiny solanums inferred from concatenated ITS and *trnK-matK* DNA regions. Numbers below branches are Bootstrap values (500 replicates). Bracketed support values at three key nodes are Bayesian posterior probabilities generated for the same groupings by Martine *et al.* (2009).

Field observations of *Solanum* sp. Litchfield provided additional features that link it with *S. dioicum*. The grey and woody stems of *Solanum* sp. Litchfield are sparsely armed (though more prickly toward the base), whereas the leaves lack armature entirely. The foliage bears a short, rusty-red tomentum and a general coloration that is light green and slightly red- or yellow-tinged. Individual plants reach a height of approximately 40 cm, with the largest stems growing to 4-5 mm in diameter. Prickles are straight, slim, and 2-3 mm long.

Like many Australian congeners (Symon 1980), *Solanum* sp. Litchfield is associated with sandstone rockpiles, growing in low outcrops in sand between the rocks and boulders. Surveys of populations on and around rockpiles led to the observation that most rockpile populations are at least partly clonal, with ramets connected via underground runners 10-12 cm below the surface. This is a habit common in *S. dioicum*, *S. petraeum*, and other similar members of the 'Kimberley Dioecious' clade (Symon 1980; Martine, pers. obs.). Much like these species, *Solanum* sp. Litchfield resprouts from runners following fire, with new post-fire growth being more vigorous and heavily armed in comparison to older growth (Martine & Vogt, pers. obs.). The three localities referenced here for *Solanum* sp. Litchfield are areas where controlled fires have been periodically set to avoid mass conflagrations, a management scheme that appears to benefit the species. Vigorous growth was witnessed where fires had recently burned (localities near Litchfield Park Road, Table 2), whereas plants appeared to be declining in vigor in unburned sites around The Lost City.

The close relationship of a dioecious *Solanum* from the Northern Territory to a group of species largely restricted to the Kimberley is interesting, but not a unique circumstance. *Solanum carduiforme*, also a member of the 'Kimberley Dioecious' clade (Figure 2), is currently known from four widely disjunct localities running from the eastern Kimberley to western Queensland, including a single population recorded by W.R. Barker and C.T. Martine in 2004 at Keep River National Park, NT. A recent flora survey at Bullo River Station in the eastern Kimberley, NT, by staff at the Northern Territory Herbarium resulted in collections of specimens that closely resemble both *S. carduiforme* and *Solanum* sp. Litchfield (I. Cowie & J. Westaway, pers. comm.). If confirmed, these would represent significant new locations for each taxon.

Although *Solanum* sp. Litchfield has been provisionally recognised as a distinct taxon by botanists at the Northern Territory Herbarium for some time, its taxonomic affinity and its reproductive biology have not previously been determined. A more complete understanding of *Solanum* sp. Litchfield and its potential designation as a new species await the collection and examination of adequate specimens, especially those with flowers and fruits. In addition, future work, including scanning electron microscope photography and greenhouse-crossing experiments, are needed to confirm that *Solanum* sp. Litchfield, like the other dioecious bush tomatoes, is functionally dioecious via the production of inaperturate pollen grains in

morphologically hermaphrodite flowers. This work necessitates collection of fruits and seeds to be used in culturing the species.

The most important work, however, will include a broad morphological and molecular survey of the taxonomically problematic *Solanum dioicum* species complex, of which *Solanum* sp. Litchfield is a member. This survey, currently underway, may provide the evidence required to describe *Solanum* sp. Litchfield as a distinct species. In the meantime, molecular recognition of *Solanum* sp. Litchfield provides evidence for its affinity to *Solanum dioicum*, broadens our understanding of the *S. dioicum* complex, and expands the known range of the 'Kimberley Dioecious' clade.

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