Phylogeny of the Australian Solanum dioicum group using seven nuclear genes, with consideration of Symon’s fruit and seed dispersal hypotheses

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Abstract

The dioecious and andromonoecious Solanum taxa (the “S. dioicum group”) of the Australian Monsoon Tropics have been the subject of phylogenetic and taxonomic study for decades, yet much of their basic biology is still unknown. This is especially true for plant-animal interactions, including the influence of fruit form and calyx morphology on seed dispersal. We combine field/greenhouse observations and specimen-based study with phylogenetic analysis of seven nuclear regions obtained via a microfluidic PCR-based enrichment strategy and high-throughput sequencing, and present the first species-tree hypothesis for the S. dioicum group. Our results suggest that epizoochorous trample burr seed dispersal (strongly linked to calyx accrescence) is far more common among Australian Solanum than previously thought and support the hypothesis that the combination of large fleshy fruits and endozoocchorous dispersal represents a reversal in this study group. The general lack of direct evidence related to biotic dispersal (epizoochorous or endozoocchorous) may be a function of declines and/or extinctions of vertebrate dispersers. Because of this, some taxa might now rely on secondary dispersal mechanisms (e.g. shakers, tumbleweeds, rafting) as a means to maintain current populations and establish new ones.

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Species (9) were placed in the more typical fleshy-fruited category, particularly in the consideration unclear by Symon (S. . . trample burrs (with fruits enclosed in prickly calyces . . . seeds shaken from fruits attached to wispy stems, e.g. S. tudunggae), or as trample burrs (with fruits enclosed in prickly calyces [Fig 1A–1C] adhering to animal fur, e.g. S. leopoldense, S. asymmetricphyllum). The dispersal mechanisms of three other taxa were hypothesized dispersal categories that can be summarized as follows: 1) pulpy/fleshy berries of various colors dispersed following ingestion (67 species; e.g. Fig 1D, 1E, 1G and 1H), 2) firm, ultimately bony berries enclosed in a calyx with unclear dispersal (perhaps ingestion) (10 species; e.g. Fig 1A), 3) smallish berries enclosed in a prickly calyx and ostensibly functioning as trample burrs on the feet of mammals (6 species; e.g. Fig 1B), and 4) a postulated set of "oddball" fruits/mechanisms not matching those above (8 species, e.g. Fig 1I).

Introduction

The large and cosmopolitan plant genus Solanum L. consists of nearly 1,400 accepted species [1], the majority of them exhibiting fleshy fruits linking them to biotic agents that disperse seeds as a consequence of frugivory [2]. Symon [3], focusing only on the ca. 90 Solanum species described for Australia at the time, defined a set of fruit morphologies that correspond to hypothesized dispersal categories that can be summarized as follows: 1) pulpy/fleshy berries of various colors dispersed following ingestion (67 species; e.g. Fig 1D, 1E, 1G and 1H), 2) firm, ultimately bony berries enclosed in a calyx with unclear dispersal (perhaps ingestion) (10 species; e.g. Fig 1A), 3) smallish berries enclosed in a prickly calyx and ostensibly functioning as trample burrs on the feet of mammals (6 species; e.g. Fig 1B), and 4) a postulated set of "oddball" fruits/mechanisms not matching those above (8 species, e.g. Fig 1I).

Fig 1. Fruit and calyx forms of selected AMT Solanum taxa.
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A fair number of the species in categories 2, 3, and 4 belong to a recently-radiated [4,5] group of “spiny solanums” (i.e. Solanum subgenus Leptostemonum, a clade of ca. 450 species with highest levels of species richness in both Old and New World Tropics) known as the “Solanum dioicum group” [6,7], a set of perhaps 30 species restricted to the Australian Monsoon Tropics (AMT) of northern Western Australia, the northern portions of the Northern Territory, and far-western Queensland. The S. dioicum group was first recognized as a subset of S. subgenus Leptostemonum (based on morphology) by Whalen [7] as a diverse group of erect to spreading shrubs and is the only group of Solanum species that includes both a large number of andromonoecious species and a set of cryptically dioecious species. Already considered unusual among Australian solanums for their breeding systems [8,9], these species are also markedly variable in morphology—with many taxa easily recognized via differences in vegetative form, habit, tomentum and armature, among other characters. Some of the greatest diversity, however, is related to fruit form and the degree to which fruits are enclosed by armed calyces, characters exhibiting particular influence on seed dispersal.

The 16 S. dioicum group taxa treated by Symon [3] include species that appear to disperse seeds via fruit fracturing (S. oedipus, S. heteropodium), “censer-like” mechanisms (described by Symon has having seeds shaken from fruits attached to wispy stems, e.g. S. tudununggae), or as trample burrs (with fruits enclosed in prickly calyces [Fig 1A–1C] adhering to animal fur, e.g. S. leopoldense, S. asymmetricphyllum). The dispersal mechanisms of three other taxa were considered unclear by Symon (S. carduiforme, S. petraeum, S. cataphractum). The remaining species (9) were placed in the more typical fleshy-fruited category, particularly in the
Calyx morphology is variable across the *S. dioicum* group, with calyces all prickly (sometimes heavily so) and accrescent to varying degrees of fruit coverage (illustrated in Fig 1). Fruits with calyces enveloping the fruit by half or less are assumed to fit into Symon’s broad category of ingested fruits, while those that are fully enveloped by calyces are assumed to be trample burrs. The exceptions to the latter condition recognized by Symon are 1) *S. tudununggae* and its “censer” mechanism, and 2) members of the *S. melanospermum* + *S. clarkiae* clade in which the accrescent calyx reflexes and presents the berries at maturity—representing a sort of pre-dispersal defense mechanism to prevent the ingestion and distribution of immature seeds (seen also in the South American [but widely naturalized] *S. sisymbriifolium* Lam.). Likewise, Symon suggested that even immature fruits without enveloping calyces are cryptically colored (being green or striped green) and exhibit greater levels of alkaloids than those that are mature (see Fig 1H), a phenomenon that is well-documented in other spiny solanums including the mainly Neotropical species of *Solanum* section *Acanthophora* [11].

Specific seed dispersal mechanisms have not been published for any of the spiny *Solanum* species in the AMT. Seeds of some taxa can survive gut passage and are germinable after ingestion by rats ex situ [12], but throughout nearly 20 years of AMT *Solanum* field observations and inspection of thousands of wild plants by Martine and colleagues, frugivory (whether by direct observation or removed fruits) has rarely been witnessed (see Table 1). Removal of similar *Solanum* fruits in other parts of the world has also been rarely recorded, although some evidence exists for *S. carolinense* L. in the US [see 13,14] and elephants and impalas have been recently identified through DNA metabarcoding as dispersers in parts of Africa [15]. While Australia lacks equivalent extant large mammalian herbivores, indigenous knowledge of the biota of the AMT does confirm that a few spiny *Solanum* species there are eaten by native mammals, especially macropods (kangaroos and wallabies). The Martu people of the western desert report hill kangaroo (*Macropus robustus* Gould) and burrowing bettong (*Bettongia lesueur* Quoy & Gaimard, now locally extinct) frugivory on *S. diversiflorum* and *S. phlomoides* (R. Bird, pers comm). Local indigenous groups from the region of Kakadu National Park (a biodiversity hotspot in far-northern Northern Territory) suggest that the fruits of a few regional endemics may also occasionally be ingested by rock kangaroos [17], but only one species from the region’s flora (*Gardinia lucata* R. Br. ex Benth., *Rubiaceae*) has been found to be ingested and effectively (although rarely) dispersed by these reclusive marsupials [18]. Most AMT *Solanum* populations we have observed either retain the majority of their fruits well into the following season or, if the fruits are abscised when mature, the berries lay in uneaten piles beneath the parent plants. Given the limited extant evidence for frugivory/dispersal, one is left to ponder whether the production of large, fleshy berries by some AMT *Solanum* represents an anachronism related to the mass extinction of nearly all large-bodied Australian animals over the last 400,000 years; or, perhaps, whether the production of such fruits is a relict of ancestral morphology and not related to biotic dispersal in Australia, at all.

Since Symon’s revision [19], many new species have been described from the region [10, 20–27]. We here revisit seed dispersal strategies among the AMT *Solanum* taxa based on the present taxonomic understanding of the group and two decades of additional observations by workers in the genus, primarily to test the hypothesis presented by Symon [3] that biotic dispersal by ingestion is the most common dispersal mechanism (with a few exceptions). Through new observations coupled with phylogenetic data, we show that not only are large, fleshy fruits less common than burr fruits in our study group, but also that they appear to represent a recent reversal in AMT *Solanum*.

**Methods**

**Taxon sampling**

Seventy-six individuals representing 50 species of *Solanum* (including roughly 25% of the presently-described *Solanum* species in Australia) were sampled from field collected dried tissue and/or fragments removed from pressed, dried herbarium specimens. Field collections were made under the following collection permits: Parks Australia RK830 and RK916, NT Parks and
Microfluidic PCR was carried out for 96 *Solanum* samples in the Fluidigm Access Array system (Fluidigm, San Francisco, California, USA) following the manufacturer’s protocols. Each of the 96 samples were amplified with the 24 primer pairs two times to completely fill the 48-well Fluidigm chip. To remove unused reagents and/or undetected primer dimers smaller than ~250 bp, each pool was purified with 0.6X AMPure XP beads (Agencourt, Beverly, Massachusetts, USA). PCR pools were analyzed in a Bioanalyzer High-Sensitivity Chip (Agilent Technologies, Santa Clara, California, USA) and standardized to 13 pM using the KAPA qPCR kit (KK4835; Kapa Biosystems, Woburn, Massachusetts, USA) on an ABI StepOnePlus Real-Time PCR System (Life Technologies, Grand Island, New York, USA). The resulting pools were multiplexed and sequenced in an Illumina MiSeq (San Diego, California, USA) to obtain 300 bp paired-end reads. Microfluidic PCR, downstream quality control and assurance, and sequencing were carried out at iBEST.
Data processing

Raw reads were filtered, trimmed, and demultiplexed by barcode and target-specific primer using dbcAmplicons ([https://github.com/msettles/dbcAmplicons [30]]) and merged using FLASH [31]. Consensus sequences for each sample in all amplicons were generated using the reduce_amplicons R script ([https://github.com/msettles/dbcAmplicons/blob/master/scripts/R/reduce_amplicons.R [25]). Raw reads have been deposited in the Short Read Archive with links to BioProject accession number SUB4712600/PRJNA498556 in the NCBI BioProject database ([https://www.ncbi.nlm.nih.gov/bioproject/].

Phylogenetic analyses

Individual loci were aligned using MUSCLE v3.8.5 [32] and adjusted manually. Phylogenetic topologies were estimated from the seven loci (summarized in Table 3) using three methods. First, gene trees for each locus were estimated using IQ-TREE version 1.5.5 [33-34] with the optimal substitution models selected by ModelFinder [35]. Clade support was determined by nonparametric bootstrapping using the ultrafast bootstrap with 1000 replicates [36]. Next, the resulting gene trees were used as input to ASTRAL-III [37] to estimate a species tree and multi-locus bootstrapping to calculate local posterior probability values for the species tree (100 replicates). Finally, we used Bayesian inference to estimate a phylogeny of a concatenated dataset using MrBayes 1.6.4 in parallel [38-40]. We applied the GTR+I+G model but allowed substitution rates for each gene to vary. The topology, branch lengths, shape, and state frequencies were unlinked. We sampled trees from two runs using eight chains (two hot, six cold) that ran for 5 X 10^6 generations with trees and model parameters sampled every 5000 generations. Convergence of the runs and stationarity were assessed for all parameters with the assistance of Tracer v.1.6.0 [41]. The Bayesian maximum clade credibility topology with posterior probabilities for each bipartition was summarized after discarding 25% of the sampled trees as burn-in using TreeAnnotator v.2.4.1 [42].

Ancestral state reconstruction

We constructed an unordered morphological character matrix in Mesquite v.3.5 [43] for calyx form at fruit maturity, using the following character states: 1) reflexed, 2) subtended/typical, and 3) enclosed/trample burr. Character states were obtained for each taxon from Symon's monograph [19] and/or field observations by the authors. We mapped calyx form onto the ASTRAL-III species tree estimate using unordered parsimony in Mesquite. The number and locations of transitions between character states was noted to determine whether calyx form is homoplasious and to better understand the associations between shifts in morphology within and among clades.

Results

Primer design and validation

MarkerMiner v.1.0 allowed for efficient design of primer pairs targeting putatively single-copy nuclear regions. We designed 48 pairs of primers for Solanum, of which 32 pairs were successfully validated and 24 were chosen for final amplification and sequencing (S1 File).

Nuclear gene sequencing

Seven of the original 48 primer pairs we attempted obtained enough sequence data and coverage for downstream use (Table 2). In total, 503 new Solanum sequences were generated and analyzed. Data from this study are available in GenBank (S2 File).

Phylogenetic analyses

Characteristics of the individual alignments used for phylogenetic analysis are summarized in Table 3. Individual gene trees estimated with IQ-TREE reveal weakly supported relationships among outgroup and ingroup clades and low overall resolution within sampled Australian Solanum [S1 Fig], trees also available as part of above referenced BioProject [SUB4687811/PRJNA498556] and via TreeBASE at http://purl.org/phylo/treebase/phylows/study/TB2:523956. No individual topologies lend support to the monophyly of the Australian species but do support some relationships within species and
among close relatives. The relationships between many species and within species that are represented by multiple individuals are unresolved by the individual genes.

The ASTRAL-III species tree analysis was informed by the seven gene trees and attributed conflicts in the data to incomplete lineage sorting. This analysis uncovered a moderately well-supported topology that is aligned with previous studies focused on the *S. dioicum* group [4,5] (Fig 2) and shows finer resolution among our ingroup taxa than does recent work sampling broadly across Old World subgenus *Leptostemonum* by Aubriot, et al. [44] in which only four *S. dioicum* group species were included (and relationships among them and to other Australian taxa largely unresolved). The ASTRAL-III topology infers four main lineages among the AMT spiny solanums (per our sampling): the Kimberley dioecious clade, the Andromonoecious bush tomatoes, the Kakadu dioecious clade, and the *S. echinatum* group. The earliest diverging of these was the Kimberley dioecious clade (local posterior probability = 0.83). The Andromonoecious bush tomatoes were found to be polyphyletic in this study due to the position of *S. oedipus*, which is sister both to the Kakadu dioecious clade and to the rest of the Andromonoecious bush tomatoes. However, the remaining species of Andromonoecious bush tomatoes form a clade (local posterior probability <0.70). The Kakadu dioecious clade is well supported (local posterior probability = 0.94), and the *S. echinatum* group is moderately well supported (local posterior probability = 0.79).

An identical overall topology was recovered by the Bayesian maximum clade credibility tree (Fig 3) which was derived from a concatenated partitioned analysis of all loci, although the posterior probabilities (pp) from this analysis vary. The Kimberley dioecious clade was less well supported (pp < 0.70), while the clade of Andromonoecious bush tomatoes (excluding *S. oedipus*), the Kakadu dioecious clade, and the *S. echinatum* group were more well supported (pp of 0.99, 1.0, and 1.0 respectively). Also worth noting is the nonmonophyly of many species for which multiple accessions were included, a pattern also apparent in the individual gene trees (S1 Fig).

Overall, the phylogenetic analyses support a close relationship between the *S. dioicum* group *sensu* Whalen [7] and the *S. echinatum* group *sensu* Bean [6] and recover with some confidence three of the five clades within the *S. dioicum* group previously identified by Martine et al. [5]: the Kimberley dioecious clade, the Kakadu dioecious clade, and the andromonoecious bush tomatoes. Of the two remaining clades not recovered here, the close relationship of the *S. melanospermum* + *S. clarkiae* clade to the bush tomatoes is inferred by the placement of the single species from the former clade in our dataset, *S. ultraspinosum*. The placement of *S. oedipus*, the single species included representing the *S. oedipus* + *S. heteropodium* clade, renders the andromonoecious bush tomato group [19] paraphyletic. Likewise, placement of the multiple morphotypes of *S. eburneum*, *S. carduiforme*, *S. dioicum*, and *S. cowiei* suggests that these species are not monophyletic as currently circumscribed and in need of revisionary study.

**Ancestral state reconstruction**

The character state reconstruction (Fig 4) supports a typical or subtended calyx as ancestral in these *Solanum* species, including the spiny *Solanum* outgroup taxa. Within the Australian *S.
diosic group, there is a transition to an enclosed or trample burr calyx morphology, which is the ancestral form in the *S. diosicum* group. There is equivocal support for a transition to reflexed calyx morphology or a reversal to the typical or subtended calyx morphology within most of the Andromonoecious bush tomatoes. *Solanum oedipus*, the andromonoecious species sister to the rest of the bush tomatoes plus the Kakadu dioecious taxa, retains the enclosed (trample burr) calyx form.

**Fig 4.** Most parsimonious ancestral state reconstruction of calyx morphology mapped onto the ASTRAL-III species tree. Clade labels follow Martine, et al. [4-5]. The *S. echinatum* group is based on Bean [6]. https://doi.org/10.1371/journal.pone.0207564.g004

**Discussion**

We present a phylogeny of the *Solanum diosicum* group derived from nuclear data mined with MarkerMiner v.1.0 using 1KP transcriptomes with sequence data generated via microfluidic PCR-based amplicon enrichment and Illumina sequencing. These data support a monophyletic assemblage that includes the *S. diosicum* group species and reveals a close alliance with the *S. echinatum* group taxa. Future phylogenomic study of these plants will target members of both groups to test the close relationship uncovered here (McDonnell & Martine, in prep). The species tree hypothesis for these groups was used to investigate the evolution of calyx form within the lineage in an effort to better understand morphological diversity and natural history of these plants.

Calyx form and dispersal type are somewhat closely correlated with breeding systems among the species in what now might be considered the “*S. diosicum + S. echinatum Group.*” Species with large, fleshy fruits and mostly non-accrescent calyces are andromonoecious, while those with putative trample burr dispersal are dioecious or hermaphrodite. The phylogeny inferred by our analyses suggests that the andromonoecious breeding system (and thus their fruit type) is derived in our study group (having arisen from either dioecy or hermaphroditism), a hypothetical sequence that runs counter to the hermaphroditism-andromonoecy-dioecy sequence proposed by previous authors [3–5,8,9,19]. While this would upend conventional understanding of breeding system evolution in this group, we acknowledge that the limitations of our data force us to maintain skepticism pending additional study. The phylogeny presented here suffers from poor resolution, which is a natural result from our use of a small number of short loci and a likely consequence of the young age of our study lineage. We believe primer design challenges, such as the initial limitations of comparing *Solanum* transcripts to *Arabidopsis* genomes with MarkerMiner 1.0, could be a cause for the failure of many primer pairs to successfully amplify loci. However, we are confident that our current species-tree hypothesis for the group is plausible and that the combination of our phylogeny with many years of field observations offers worthwhile insight into the role of fruit and calyx form in seed dispersal among Australian *Solanum*.

**Fruit/calyx form and seed dispersal**

Despite decades of study, the natural history of solanums in the AMT is still quite poorly understood—particularly with regard to plant-animal interactions including pollination [49] and seed dispersal [3]. Nearly all of the AMT *Solanum* species produce fleshy berries; and although traditional knowledge suggests that some animals do eat *Solanum* fruits [16,17] and may disperse seeds, no effective biotic seed dispersal interaction has yet been identified for a single species within the group. Still, one might infer that biotic dispersal following frugivory is a likely means by which many AMT solanums colonize new areas. Symon [3] suggested that birds, mammals, and lizards all play a role in endozoochorous dispersal of *Solanum* seeds in Australia; meanwhile, epizochorous and non-biotic modes of dispersal (including wind, water, and mechanical modes) were hypothesized to play a secondary role.

As Symon [3] pointed out, the interaction of fruit form and calyx morphology in seed dispersal appears to be significant. Based on a combination of field and greenhouse observations, herbarium study, experimental work, description of new species, and molecular phylogenetics over the last 15+ years, we suggest that for the *S. diosicum* group, where many species exhibit accrescent fruiting calyces for all or part of fruit/seed development, calyx form may be even more important than previously assumed. Specifically, current understanding of diversity in this group (including the many new species described since Symon’s 1981 monograph [19]) suggests that burr-fruited species (with accrescent prickly calyces that remain closed after fruit maturity) are more common than calyces that only partly cover fleshy fruits. Whereas Symon hypothesized that six species across all of *Solanum* in Australia dispersed by trample burrs, we suggest that this number is closer to 18 in the *S. diosicum* group sensu stricto alone (Table 1). Likewise, the
phylogenetic analyses infer that the *S. echinatum* group sensu Bean [6] (here represented by *S. echinatum*, *S. raphiotes*, and *S. lucani*) is nested in the *S. dioicum* group with which it shares the enclosed trample burr morphology (or, at least, an accrescent prickly calyx). Recent work by Bean [48] suggests that species diversity in the *S. echinatum* group is greater than currently described and, therefore, so is the presence of trample burr dispersal in that clade.

For the species with accrescent calyces, the calyx appears to play two significant roles. One function, for most species, is in fruit and seed dispersal. The other function is likely that of a "pre-dispersal defense," or the prevention of unripened fruits from being removed and ingested before the seeds are mature and germinable. In the case of some species, such as *S. oscuricuuentum*, readiness of seeds appears to correspond with whole fruits (with enveloping prickly calyx) dropping off the plants entirely [26]. In perhaps the most elegant example of pre-dispersal defense, the members of the *S. melanosperrnum* + *S. clarkiae* Clade (those two species plus the unsampled recently-described *S. ultraspinosum* and *S. apodophyllum* [21]) enclose their unripened fruits in heavily-armed calyces before presenting them, still attached, with reflexed calyces at fruit maturity (Fig 1E)—a phenomenon also shared by a number of other spiny solanums (e.g., *S. sisymbrifolium*). Pre-dispersal defense via secondary chemistry among Australian species with non-accrescent calyces was proposed by Symon [3], and has been well-studied in other *Solanum* clades (e.g. [11,13,14,47]), with green striping on fruits produced by most of the non-accrescent species presumed to be both a warning signal and a camouflage via cryptic coloration against green foliage (Fig 1H).

In addition to an assumed greater role for trample burr dispersal, our observations have also revealed that some of the secondary abiotic dispersal strategies proposed by Symon [3] are more common than previously imagined. Intriguingly, some of these seem to function as dispersal insurance for when fleshy fruits are not removed by frugivores and, instead, are left to dry out and eventually break apart. The mechanical "censer" dispersal Symon [3] ascribed to a single Australian species, *S. tudununggae*, might be better broadly described as a "shaker" mechanism inclusive of other species in which this has been observed. A prime example is *S. chippendaeli*, where hundreds of individuals (near Taylor Creek, Northern Territory) were witnessed bearing the previous years' fruits which, now dry and brittle, were dropping loose seeds from open fruit wall cavities (Fig 1J with the slightest touch or breeze (Martine, pers. obs.). Even in cultivation, *S. chippendaeli* fruits age differentially—with some areas of the fruit wall breaking down much quicker than others do as fruits pass beyond maturity. Similar shaker dispersal has also been seen in *S. eburneum* and may apply to what Symon [13] described as "fracturing" in *S. oedipus* and *S. heteropodium*—as well as what Knapp et al. [48] describe for the New World *S. elaeagnifolium* clade.

Symon [3] suggested that *S. pugiunculiferum*, a species found in tidal mudflats that is not a member of the *S. dioicum* group, might be described as using tumbleweed dispersal—with whole plants (with fruits retained) breaking off and tumbling during the AMT dry season or rafting on water during the wet season. Our recent observations of *S. melanosperrnum* in the Northern Territory's highly-dynamic Robinson and MacArthur River systems suggest that this species moves about in a similar fashion, even though it is also an obviously reflexed-calyx fruit-presenter (Fig 1D). Shrubs holding onto hundreds of ripened and post-mature fruits have been found tossed along the deep sandy banks of these rivers like so much flotsam and jetsam. Water might likewise disperse some fruits that mature as bony and hollow, particularly when enclosed in calyces providing additional surface area among the prickles. We have observed the enclosed fruits of *S. oscuricuentum*, *S. cardiforme*, and *S. echinatum* piled up in debris lines deposited by sheet wash from the previous season’s monsoon rains, a phenomenon common in arid zones [49–51].

Still, the broad observation that we have made is that, regardless of the "intended" mode of dispersal, precious few fruits appear to be carried off (whether internally or externally) by a biotic disperser and have thus become dependent on (seemingly) secondary abiotic forms of dispersal. Our unpublished observations growing thousands of plants from seed in cultivation has shown that untreated seeds are able to germinate (even though rates are much higher following gibberellic acid treatments), suggesting that gut passage is not a dispersal requirement—even if it might offer some obvious benefits. Martine and Anderson [9] proposed that AMT *Solanum* might be engaged in a type of secondary dispersal by which macropods provide short-distance seed dispersal through a three-step process: fruit ingestion, "fecal seed storage" (after seeds are moved with animals to their denning sites and defecated), and "seasonal redispersal" via wet season rains. The general concept still applies to plant species producing trample burrs and may be even more likely than a scenario dependent on ingestion, given the hypothesized prevalence of epizoochy in the group.

The notion that the extinction of many of Australia’s medium- to large-bodied vertebrates in the last 40,000 years [52] may have rendered some primary dispersal mechanisms anachronistic is worth considering. One might imagine that the lack of current data related to effective seed dispersal is a simple reflection of the dearth of potential dispersers in the AMT, a factor made limiting by past megafaunal extinctions in Australia. However, no fossil record exists for large-scale megafauna extinctions over much of what is now the AMT [53,54]. Meanwhile, fossil records provide ample evidence for extinctions in southwestern [55] and southeastern [56] Australia; the assumed range of megafauna reached northward primarily along the western and eastern coasts [55,56], perhaps because of inconsistent availability of water in central and north-central regions.
In the areas of the continent where evidence is lacking for past megafauna abundance, relatively few extant plant species produce fruits fitting the typical profile of the megafauna dispersal system (large size, tough endocarp, and soft pulp [57]). Over much of interior and northern Australia, Pleistocene seed dispersal may have been left to macropods and other browsers (both extinct and extant), who may have been infrequently and opportunistically frugivorous—but who, largely, may have carried fruits/seeds in their fur. Trample burr fruit/calyx morphology may thus have been an advantage in an ecosystem lacking abundant large frugivores—and continues to be an advantage today.

Even where the megafauna syndrome is present in Australia it appears to not parallel the large-fruited big-tree version of Janzen and Martin [57]. Instead, plants exhibit a suite of growth forms that would have allowed shrubbier plants to resist browsing by large herbivorous animals—including physical and chemical defenses—while also producing fruits and seeds adapted for vertebrate dispersal [54], including trample burrs. In the present day, many of the remaining small- and medium-sized mammals (as well as frugivorous birds) throughout the AMT have recently experienced and continue to face declines [58-60]. This likely places many biotically-dispersed plants at risk and may put those species with potential secondary mechanisms (e.g. shakers or tumbleweeds) at a short-term advantage. In the long run, however, local extirpations and wholesale extinctions of biotic dispersers are likely to have consequences for the plants linked to them. The observations of the Martu people [16] and R. Bird (pers. comm) at the boundary of the Little Sandy and Great Sandy Deserts are seemingly telling in this regard: one confirmed Solanum frugivore, the burrowing bettong (Bettongia lesueurii), is now locally extinct; and the other, the likely-opportunistic hill kangaroo (Macropus robustus), handles the fruits of two species in such a way that the bitter seeds are avoided entirely. Demographic analyses (Cantley, et al., in prep) infer that several AMT Solanum species have undergone recent genetic bottlenecks, a trend that would likely continue should their biotic dispersers also go the way of the burrowing bettong.

The evolutionary development of calyx morphology is one of many exciting areas of current research in Solanaceae [61, 62], especially in the context of seed dispersal biology. Among our Solanum study species, calyx form exhibits particular lability—with broad occurrence of derived accrescence/enclosure plus a reversal to the Solanum ancestral state of a non-enveloping calyx associated with fleshy berries. Further research employing larger molecular datasets and increased sampling (McDonnell and Martine, in prep) will shed greater light on the evolution of this and other fascinating elements associated with the reproductive biology of the S. doicium + S. echinatum group.

Supporting information

S1 Fig. Individual gene trees for each of the seven loci as estimated by IQ-TREE. Values at nodes reflect bootstrap support. https://doi.org/10.1371/journal.pone.0207564.s001 (PDF)

S1 File. List of specimen vouchers and GenBank numbers for sequences used in this study. For each accession, information is as follows: collector and collector number, DNA isolate name, locality, date of collection, acronym for herbarium (in parentheses) where voucher is held, and gene regions recovered for that accession. GenBank accession numbers for gene regions used in this study are listed for each taxon in the following order, with a dash (—) inserted where a region was not recovered: SOL3, SOL8, SOL9, SOL12, SOL14, SOL15, SOL16. https://doi.org/10.1371/journal.pone.0207564.s002 (PDF)

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