Phylogenetic placement and the timing of diversification in Australia's endemic *Vachellia* (Caesalpinioideae, Mimosoid Clade, Fabaceae) species

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Abstract.   The genus *Vachellia* Wight & Arn. has a pantropical distribution, with species being distributed through Africa, the Americas, Asia and Australia. The relationships among the lineages from Africa and America are well understood, but the phylogenetic placement and evolutionary origins of the Australian species of *Vachellia* are not known. We, therefore, sequenced four plastid genes from representatives of each of the nine Australian species of *Vachellia*, and used Bayesian inference to assess the phylogenetic placement of these lineages, and a relaxed molecular clock to assess the timing of diversification. The Australian species of *Vachellia* form a well-supported monophyletic clade, with molecular-dating analysis suggesting a single dispersal into Australia 6.5 million years ago (95% range 13.9–2.7 million years ago). Diversification of the Australian clade commenced more recently, *c.* 3.1 million years ago (95% range 9.2–1.2 million years ago), perhaps driven by the increased aridification of Australia at this time. The closest relatives to the Australian *Vachellia* were not from the Malesian bioregion, suggesting either a long-distance dispersal from Africa, or two separate migrations through Asia. These results not only improve our understanding of the biogeography of *Vachellia* species, but also have significant implications for the biological control of invasive *Vachellia* species in Australia.

Introduction

The disjunct pantropical distributions of some plant groups have long been a topic of interest for biogeographers (de Candolle 1820; Darwin 1859), with the extensive breaks in distribution being traditionally attributed to long-distance transoceanic dispersal (Darwin 1859; Nelson 1978). After the universal acceptance of plate tectonic theory in the 1960s, vicariance (the historic fragmentation of the ancient landmass of Gondwana) then became the predominant explanation for these patterns (Raven and Axelrod 1972; Rosen 1978). The development and refinement of phylogenetic techniques and molecular-dating analysis in the past 2 decades has provided researchers with new tools for understanding large range disjunctions in plant species (Renner 2005). Using these techniques, researchers have discovered that phylogenetic divergences and disjunct distributions of many plant taxa post-date the fragmentation of Gondwana (Cook and Crisp 2005; Bouchenak-Khelladi *et al*. 2010; Bartish *et al*. 2011), suggesting that long-distance transoceanic dispersal has played a significant role in the pantropical distributions of many plant species (Gillespie *et al*. 2012).

Vachellia Wight & Arn. is a large genus (161 described species) of leguminous flowering trees and shrubs, sometimes

called Shittah trees. *Vachellia* was previously classified within the genus *Acacia* Mill. *sensu lato*, as subgenus *Acacia*, but morphological and genetic data (see Maslin *et al*. 2003) led to them being raised quite recently to the genus level (Orchard and Maslin 2003). The genus *Vachellia* has a broad pantropical distribution (Fig. 1), with 73 species having been described from Africa, 60 from the Americas, 36 from southern Asia and 9 species from the tropical north of Australia (Pedley 2002; Orchard and Maslin 2003). Previous phylogenetic studies of *Vachellia* have suggested that the genus diverged from its sister clade (containing *Senegalia* Raf., *Mariosousa* Seigler & Ebinger and *Acaciella* Britton & Rose) *c.* 40 million years ago (Bouchenak-Khelladi *et al*. 2010; Miller *et al*. 2013). This split post-dates the fragmentation of the supercontinent Gondwana, suggesting that long-distance transoceanic dispersal has played a significant role in the contemporary pantropical distribution of *Vachellia* (Bouchenak-Khelladi *et al*. 2010). Indeed, the subsequent radiation within the genus started more recently, *c.* 18 million years ago, with the African–Asian clade diverging from the American clade *c.* 17–13 million years ago (Bouchenak-Khelladi *et al*. 2010; Miller *et al*. 2013).

Seeds of *Acacia s.l.* species are commonly dispersed on a local scale by mammalian or ant vectors (Miller 1995; Pascov *et al*.
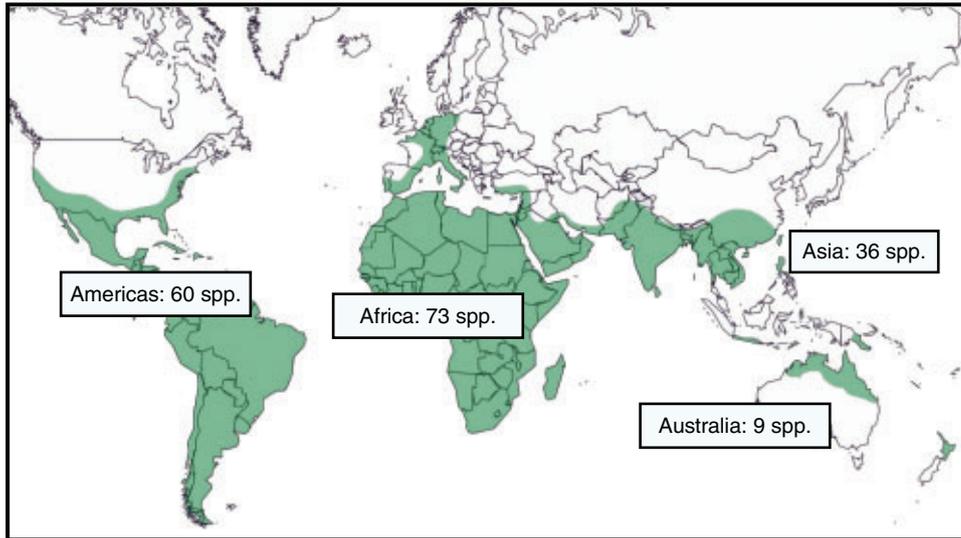
Fig. 1. Global distribution of *Vachellia* species. The invasive distributions of *V. farnesiana, V. karroo* and *V. nilotica* in Australia have not been included so that the distribution of native Australian *Vachellia* species is clear. Distributions were derived from occurrence data published on www.gbif.org (accessed 30 September 2018).

2015). However, the vector(s) for long-distance dispersal are not well understood. Transoceanic dispersal of species has been believed to be human-mediated in some instances (e.g. *A. koa* A.Gray; Brown *et al.* 2012), which may explain the cosmopolitan distribution of species such as *V. farnesiana* (L.) Wight & Arn. (Bell *et al.* 2017). The seeds of some Mimosoid species are long-lived and can remain viable even after 1 decade of immersion in salt water, potentially enabling long-distance dispersal on ocean currents (New 1984). Migratory birds have also been suggested as possible dispersal vectors for seeds in several taxa, including plants in the closely related genus *Acacia sensu stricto* (Le Roux *et al.* 2014; Viana *et al.* 2016), but their role in moving *Vachellia* seeds has never been demonstrated or otherwise established.

The phylogenetic relationships among the African (including Madagascan), Asian and American species of *Vachellia* have been characterised previously (Kyalangalilwa *et al.* 2013; Boatwright *et al.* 2015), but the phylogenetic placement of the Australian species has not yet been assessed. How these *Vachellia* species arrived in Australia remains unknown, as does the likely time of their arrival, or even whether they represent a monophyletic assemblage. Placing the Australian endemic species of *Vachellia* within phylogenetic context will contribute to the broader body of work concerning the biogeography of *Vachellia*, but also has important implications for the biological control of pest species of *Vachellia* in Australia. Three species, namely *V. nilotica* (L.) P.J.H.Hurter & Mabb., *V. farnesiana* and *V. karroo* (Hayne) Banfi & Galasso, are considered invasive weeds in Australia, with *V. nilotica* having been the target of a decades-long biocontrol program (Palmer *et al.* 2012; Hansford 2015). As host-specificity testing of biocontrol agents requires the selection of plants closely related to the target (McEvoy 1996), establishing the phylogenetic placement of the nine endemic Australian species of *Vachellia* will help inform future biological control programs targeting pest species of *Vachellia* in Australia.

In this study, we use four plastid gene regions (*mat*K, *rbc*L, *trn*L–*trn*F intergenic spacer, and *trn*H–*psb*A intergenic spacer) to assess the phylogenetic placement of the Australian species of *Vachellia*. These regions have previously been used to assess phylogenetic relationships among Mimosoid lineages (see Newmaster and Subramanyam 2009; Bouchenak-Khelladi *et al.* 2010; Kyalangalilwa *et al.* 2013; Boatwright *et al.* 2015), allowing us to add to pre-existing datasets. In addition, we use a relaxed molecular clock to estimate when the Australian species diverged from their closest relatives. We also discuss the importance of these results for understanding the biogeography of the genus *Vachellia*, and the implications for biological control of invasive species of *Vachellia* in Australia.

Materials and methods

Taxon sampling

Dried leaf material from the nine endemic Australian species of *Vachellia* (*V. bidwillii* (Benth.) Kodela, *V. clarksoniana* (Pedley) Kodela, *V. ditricha* (Pedley) Kodela, *V. douglasica* (Pedley) Kodela, *V. pachyphloia* (W.Fitzg.) Kodela, *V. pallidifolia* (Tindale) Kodela, *V. suberosa* (A. Cunn ex Benth.) Kodela, *V. sutherlandii* Kodela, and *V. valida* (Tindale & Kodela) Kodela) was obtained from specimens held at the Queensland Herbarium (Table S1, available as Supplementary material to this paper).

DNA extraction, amplification and sequencing

DNA was extracted from leaf samples by using the CTAB extraction protocol (Doyle and Doyle 1987), followed by a do-it-yourself spin-column extraction (using the protocol of Ridley *et al.* (2016)). The following four plastid DNA regions were amplified and sequenced: the *trn*L–*trn*F intergenic spacer (primers *trn*L-c and *trn*L-f; Taberlet *et al.* 1991), *rbc*L (primers *rbc*La-F and *rbc*La-R; Levin *et al.* 2003; Kress *et al.* 2009), *mat*K

(primers *mat*K-1RKIM-f and *mat*K-3FKIM-r; Kuzmina *et al*. 2012), and the *trn*H–*psb*A intergenic spacer (primers *trn*Hf and *psb*A3_f; Sang *et al*. 1997; Tate and Simpson 2003). Polymerase chain reactions (PCRs, 12 μL) contained 7.6 μL of water, 2.0 μL of 5× buffer (Bioline, London, UK), 0.24 μL each of 10 mM forward and reverse primer, 0.08 μL of *Taq* polymerase (Bioline) and 2 μL of DNA template. Cycling conditions for all genes were as follows: 3 min at 95°C, followed by 40 cycles of 95°C for 20 s, 50°C for 30 s and 72°C for 30 s, with a final extension step of 72°C run for 7 min. PCR product that successfully amplified was cleaned by adding 1 μL of exonuclease I and 1 μL of antarctic phosphatase per sample, and then sequenced in both directions by Macrogen (Seoul, South Korea).

Phylogenetic analysis

Complementary sequences were assembled in Geneious (ver. 7.0.6, Biomatters, Auckland, New Zealand, see https://www. geneious.com/, accessed 13 June 2017; Kearse *et al*. 2012). Sequences from 16 endemic Australian specimens of *Vachellia* were then aligned with homologous regions from 56 additional *Vachellia* species downloaded from GenBank (see Table S2, available as Supplementary material to this paper), along with the following four outgroups: *Acacia mearnsii* De Wild, *Albizia kalkora* (Roxb.) Prain, *Leucaena leucocephala* Lam (de Wit.) and *Senegalia senegal* (L.) Britton, using the MUSCLE plugin (R. Edgar, see www. drive5.com/muscle, accessed 13 June 2017; Edgar 2004). JModelltest (ver. 2.1.2, D. Darriba, see https://github.com/ ddarriba/jmodeltest2, accessed 30 November 2017; Darriba *et al*. 2012) was used to select the best model of sequence evolution for each plastid region. The four plastid genes were concatenated into a single dataset, because, individually, they exhibit low levels of sequence divergence, and a visual inspection detected no well supported incongruence among individual plastid-gene trees (not shown).

Phylogenetic relationships were assessed using Bayesian inference in MrBayes (ver. 3.2.6, C. Zhang, J. Huelsenbeck, P. van der Mark, F. Ronquist and M. Teslenko, see http:// nbisweden.github.io/MrBayes/, accessed 24 February 2018; Huelsenbeck and Ronquist 2001). We coded gaps (indels) as missing characters, and excluded any regions of ambiguous alignment (260 bp in total, see Table S3, available as Supplementary material to this paper). The concatenated dataset was partitioned by DNA region, allowing independent estimation of parameters for each partition. Four Markov chains were run for 10 million generations, with chains sampled every 200 generations (the first 5000 trees were discarded as 'burn-in'). We used Tracer (ver. 1.7.0, A. Rambaut, see http://tree.bio.ed.ac. uk/software/tracer/, accessed 14 March 2018; Rambaut *et al*., 2018) to monitor convergence of all parameters to ensure burn-in was sufficient, and effective sample size was greater than 200 for each prior.

In addition, maximum-parsimony (MP) analyses were conducted in PAUP* (ver. 4.0b10, D. L. Swofford, Sinauer Associates, Sunderland, MA, USA, see https://paup. phylosolutions.com/, accessed 20 February 2018; Swofford 2003). The heuristic searches employed 1000 random taxon addition sequences, with the tree bisection–reconnection branch-swapping algorithm being used. A strict-consensus phylogeny was calculated from the set of most parsimonious trees. Support for individual branches was estimated using non-parametric bootstrapping (Felsenstein 1985) with 1000 heuristic replicates.

Molecular-dating analysis

Molecular dating was performed on the Bayesian consensus tree in BEAST (ver. 1.10.4, BEAST Developers, https://beast. community/, accessed 17 March 2018; Bouckaert *et al*. 2014), using the Yule speciation model. Because a likelihood ratio test indicated that the molecular data were not clock-like, dating was conducted using an uncorrelated lognormal relaxed molecular clock (Drummond *et al*. 2006). The dataset was partitioned by DNA region (see Table S3, available as Supplementary material to this paper). The analysis was run five times, with 10 million generations per run, and the five separate runs were combined using LogCombiner. As there were no appropriate fossil samples of *Vachellia* for calibration, we used a secondary calibration point for the crown-age divergence date of *Vachellia*. The estimated divergence dates of *Vachellia* have been remarkably concordant among recent studies, with an average crown age of between 17.2 and 13.7 million years (Bouchenak-Khelladi *et al*. 2010; Gómez-Acevedo *et al*. 2010; Miller *et al*. 2013). To incorporate the uncertainty of these estimates, we used a normal prior for the crown age of *Vachellia*, with a mean of 15 million years, and a standard deviation of 3 million years, chosen so that 95% of the prior probability density was between 20 and 10 million years. A maximum clade credibility tree was generated in TreeAnnotator and visualised in FigTree (ver. 1.4.2, A. Rambaut, see http://tree.bio.ed.ac.uk/software/ figtree/, accessed 24 June 2018).

Results

Data characteristics

All new sequences obtained in this study have been submitted to GenBank (accession numbers MK923581–MK923636; Table S2), with the aligned datasets lodged in TreeBASE. Following concatenation of the four plastid regions, the edited DNA alignment was 2997 bp. Sequence statistics and substitution models are provided in Table S3.

Phylogenetic analysis

Bayesian inference (Fig. S1, available as Supplementary material to this paper) and MP analysis (Fig. S2, available as Supplementary material to this paper) supported the monophyly of the genus *Vachellia*, and returned broadly concordant relationships across the depth of the phylogeny. We use the Bayesian phylogeny as the basis for interpreting and discussing the results. There were four distinct African–Asian clades, and two distinct American clades (Fig. 2, S1). Neither the African–Asian nor American species were monophyletic; however, the majority of the African taxa were included in a well-supported clade that also includes the Australian species (Fig. 2, S1). The nine endemic Australian species of *Vachellia* formed a well-supported (pp = 1.00) monophyletic clade, sister to Africa Clade A (Fig. 2). Relationships within clades were poorly supported. There were only six parsimony-informative sites
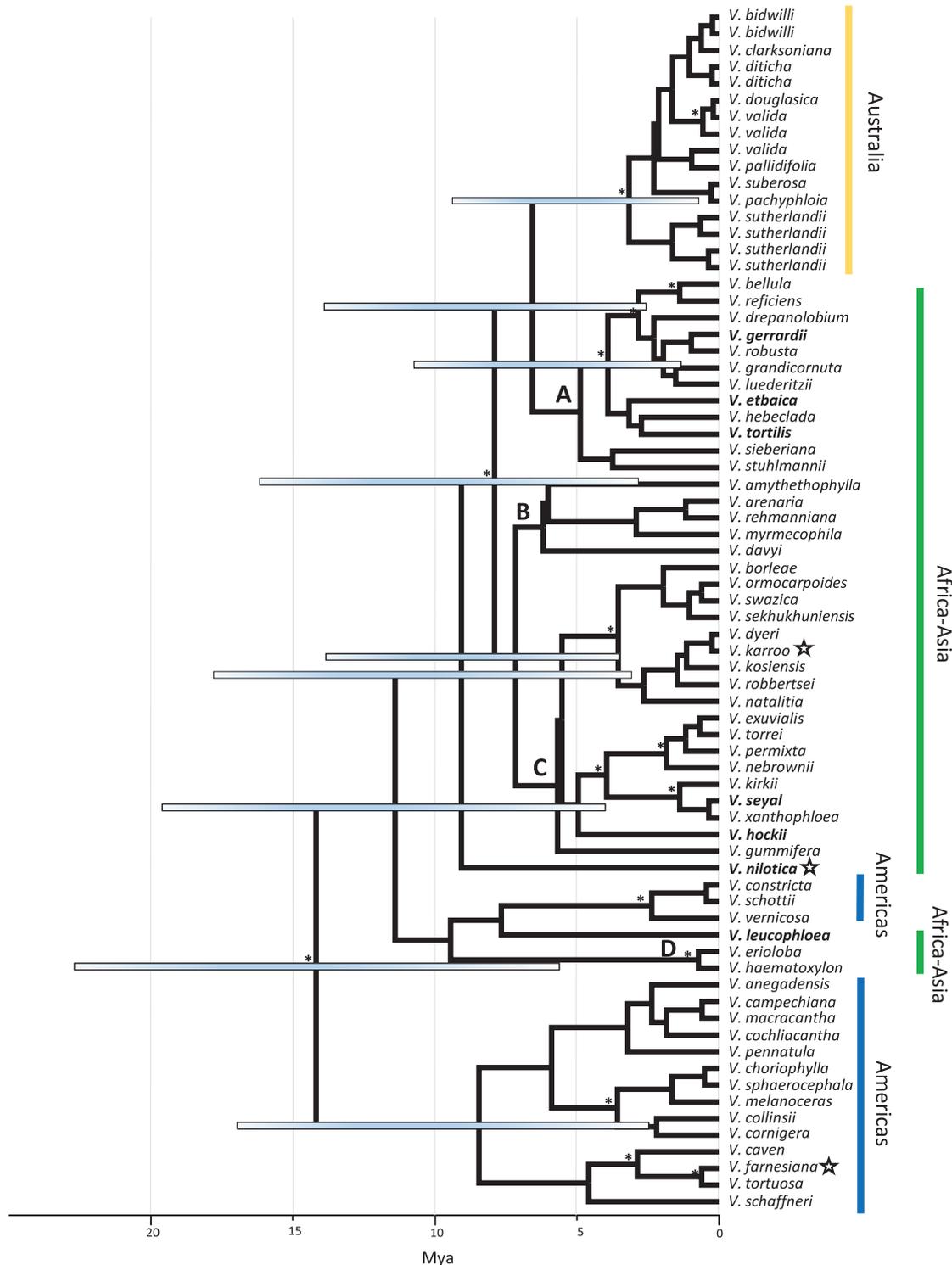
Fig. 2. Bayesian phylogenetic tree of the relationships among *Vachellia* species, based on four plastid regions. Posterior probabilities exceeding 0.90 are indicated by an asterisk above each node. Horizontal bars at nodes indicate 95% posterior probability of the age of the node. Outgroups are removed for diagrammatic clarity. The names of *Vachellia* species that are invasive in Australia are indicated with a star. Most species in the African–Asia clades are native only to Africa, apart from *V. gerrardii*, *V. etbaica*, *V. tortilis*, *V. seyel*, *V. hockii* and *V. nilotica* (all native to both Africa and Asia) and *V. leucophloea* (native to Asia). These species are indicated in bold. The four distinct African–Asian clades are labelled with a letter above the node.

across the species in the Australian endemic clade. Consequently, only one node in the Australian clade has strong support, relating two specimens of *V. valida* with *V. douglasica* (Fig. 2, S1).

The three species of *Vachellia* that are invasive in Australia (*V. karroo*, *V. nilotica* and *V. farnesiana*) are placed in different clades from one another (Fig. 2). *Vachellia karroo* is placed in Africa–Asia Clade C, *Vachellia nilotica* placed sister to African–Asia Clades A, B and C, and *V. farnesiana* is supported as the sister taxon to *V. tortuosa* in the large American clade (Fig. 2). Significantly, none of the invasive *Vachellia* species is in the clade containing the Australian endemic species, nor in the Africa–Asia Clade A (which is sister to the Australian clade).

Molecular-dating analysis suggested that the Australian *Vachellia* clade diverged from Africa–Asia Clade A *c.* 6.5 million years ago (95% range 13.9–2.7 million years ago). Speciation within the Australian clade commenced *c.* 3.1 million years ago (95% range 9.2–1.2 million years ago), and has continued through the Quaternary period. The large clade of American species diverged *c.* 14.2 million years ago (95% range 22.6–5.9 million years ago), and began to diversify *c.* 8.1 million years ago (95% range 17.1–3.2 million years ago). Speciation within the main African clade commenced at *c.* 9 million years ago (95% range 17.3–3.6 million years ago).

Discussion

Our results have demonstrated that the endemic Australian species of *Vachellia* form a monophyletic clade, being consistent with a single introduction into Australia *c.* 6.5 million years ago (Fig. 2). Unexpectedly, the Australian clade was more closely related to African–Asian Clade A (which contains no species from Southeast Asia), than to Southeast Asian species such as *V. nilotica* (found as far east as Myanmar) and *V. leucophloea* (Roxb.) Maslin, Seigler & Ebinger (the species of *Vachellia* included in the present study that extends into Malesia, although there are others; Maslin *et al.* 2013). This suggests that *Vachellia* may have dispersed into Australia from Africa as a result of transoceanic dispersal, perhaps by island hopping via Madagascar or other islands in the Indian Ocean, as has been suggested for other plant genera (e.g. *Adansonia* L., *Bridelia* Willd. and *Gaertnera* Lam.; Baum *et al.* 1998; Malcomber 2002; Li *et al.* 2009). Indeed, the sole Madagascan species in our study (*V. bellula*) was included in Clade A, supporting this hypothetical dispersal route (Fig. 2). However, the potential vector for this dispersal route remains unclear. There are no known migratory bird routes that are current between Africa and Australia, making an avian vector unlikely (Li *et al.* 2009).

The northern distribution of the nine endemic Australian species of *Vachellia* (Fig. 1) is more consistent with an introduction through the Malesian bioregion (Crisp and Cook 2013), with this biogeographic pattern being also observed in other plant taxa (e.g. Aglaieae and *Livistona* R.Br.; Muellner *et al.* 2008; Crisp *et al.* 2010). Several species of *Vachellia* are found through the Malesian bioregion (e.g. *V. tomentosa* (Rottler) Maslin, Seigler & Ebinger and *V. leucophloea*; Maslin 2008), although they extend only as far south as

Timor. If the ancestors of the Australian *Vachellia* species did arrive in Australia via the Asian land route, we would have predicted that the Malesian species *V. leucophloea* would have been sister to the Australian clade, but this was not the case (Fig. 2). However, an Asia dispersal route remains a possibility if there were multiple radiations of *Vachellia* through southern Asia and the Malesian biogeographic region, and the ancestor of the Australian species was not included in this study. Future research should involve sequencing additional Malesian and southern Asian *Vachellia* species (because there is currently only a single Malesian species of *Vachellia* on GenBank), because this may provide a clearer understanding of the provenance of Australian *Vachellia*.

Despite diverging from the *V. tortilis* clade *c.* 6.5 million years ago, subsequent speciation within the Australian *Vachellia* clade did not commence until *c.* 3.1 million years ago (Fig. 2). Towards the end of the Pliocene, the Australian continent became increasingly more arid as it drifted north towards Asia (Fujioka *et al.* 2005; Byrne *et al.* 2008). With this aridification came a monsoonal climate that is still present in Australia today, and an increase in seasonal fires (Bowman *et al.* 2010). The aridification of the Australian continent towards the end of the Pliocene era reputedly was responsible for extensive speciation in several large Australian genera at the time, including *Acacia s. s.* and *Eucalyptus* (Gill 1975; Ross 1981). The timing of this aridification matches the diversification within the Australian *Vachellia* clade, suggesting that drying conditions and the onset of the current Australian monsoon climate may have also driven diversification within this group. Furthermore, more frequent fires may also have driven speciation of the Australian *Vachellia* clade, because the seeds of many extant Mimosoid species are either fire-resistant or benefit from fires (Sabiiti and Wein 1987; Hodgkinson and Oxley 1990).

Our findings have additional implications for the biological control of the three invasive *Vachellia* species found in Australia. None of these invasive species is a particularly close relative of the Australian *Vachellia* species (Fig. 2). Consequently, the risk of herbivory on the native Australian species by potential biocontrol agents is not high, because the level of phylogenetic relatedness between target and non-target plants is considered to be positively correlated with the risk of non-target impacts (Julien *et al.* 2008). Native Australian *Vachellia* species should, nevertheless, be included in host lists for host-specificity testing of agents, but their phylogenetic separation from the three invasive species is an encouraging sign for finding host-specific herbivores for introduction against the three invasive species of *Vachellia* in Australia.

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