

Relatedness defies biogeography: the tale of two island endemics (*Acacia heterophylla* and *A. koa*)

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Summary

- Despite the normally strong link between geographic proximity and relatedness of recently diverged taxa, truly puzzling biogeographic anomalies to this expectation exist in nature.
- Using a dated phylogeny, population genetic structure and estimates of ecological niche overlap, we tested the hypothesis that two geographically very disjunct, but morphologically very similar, island endemics (*Acacia heterophylla* from Réunion Island and *A. koa* from the Hawaiian archipelago) are the result of dispersal between these two island groups, rather than independent colonization events from Australia followed by convergent evolution.
- Our genetic results indicated that *A. heterophylla* renders *A. koa* paraphyletic and that the former colonized the Mascarene archipelago directly from the Hawaiian Islands ≤ 1.4 million yr ago. This colonization sequence was corroborated by similar ecological niches between the two island taxa, but not between *A. melanoxylon* from Australia (a sister, and presumed ancestral, taxon to *A. koa* and *A. heterophylla*) and Hawaiian *A. koa*.
- It is widely accepted that the long-distance dispersal of plants occurs more frequently than previously thought. Here, however, we document one of the most exceptional examples of such dispersal. Despite c. 18 000 km separating *A. heterophylla* and *A. koa*, these two island endemics from two different oceans probably represent a single taxon as a result of recent extreme long-distance dispersal.

Introduction

Understanding the historic biogeographic processes that shaped current species distributions, and thus the evolution of different biotas, has become a central theme in ecology and is informative on how biodiversity is generated and maintained (Cavender-Bares *et al.*, 2009). Opportunities for speciation by means of geographic isolation are contributing factors that made Darwin (1859) first realize the crucial link between evolution and geography. Not surprisingly, the relationship between geographic proximity and the relatedness of recently diverged taxa can be quite strong. However, this link is often violated by, amongst others, local extinctions of some (but not all) taxa and re-colonization/dispersal (Cunningham & Collins, 1998), long-distance dispersal events (Waters *et al.*, 2013) and extremely widely distributed taxa (e.g. Harrington & Gadek, 2009), sometimes making inferences on biogeographic histories problematic.

The distribution of island biotas presents fascinating opportunities to study biogeographic patterns and processes. The theory of island biogeography predicts that insular biodiversities are

mainly products of immigration, extinction, an island's size, and its proximity to the nearest mainland regions (MacArthur & Wilson, 1967; but also see Whittaker *et al.*, 2008 and references therein). The supposed link between the proximity to mainland source regions and insular biodiversity may stem from the fact that arrivals of new species are a result of rare long-distance dispersal. Such long-distance dispersal events can often lead to the exceptional radiation of insular floras, often leading to the over- or under-representation of certain taxa compared with regional species pools (Wagner & Funk, 1995; de Queiroz, 2005; Warren *et al.*, 2010). Although the nearest continents or landmasses are often implicated as sources of island taxa, the roles of transoceanic pathways and extreme long-distance dispersal have been strongly underestimated in shaping insular biodiversities (de Queiroz, 2005, 2014; but see Birch & Keeley, 2013; Patiño *et al.*, 2013).

Truly puzzling and curious examples of biogeographic anomalies exist in nature. For example, the diverse genus *Acacia* Mill. (*sensu stricto*; previously grouped in *Acacia* subgenus *Phyllodineae*) consists of c. 1045 species, most confined to Australia, with a few

taxa found in South-East Asia and Oceania (Brown *et al.*, 2012). Two particularly peculiar extra-Australian taxa are the closely related island endemics *Acacia heterophylla* from the Mascarene archipelago (restricted to Réunion Island) in the Indian Ocean and *Acacia koa* found in the Hawaiian archipelago in the Pacific Ocean. Not only is this geographic disparity truly remarkable, with *c.* 18 000 km separating these two archipelagos, but what makes it even more interesting is that these two species are considered as each other's closest living relatives (Brown *et al.*, 2012). Indeed, morphologically and ecologically, these two species are so similar (Geesink & Korner, 1989) that they have been considered as a single species by several authors (Bentham, 1875; Pedley, 1975; St John, 1979; see Fig. 1).

Ecologists have long speculated about the unusual distribution of *A. heterophylla* and *A. koa*, which remains an enigma (but see Brown *et al.*, 2012). Both species are closely related to the Australian Blackwood tree, *A. melanoxylon* (Murphy *et al.*, 2010; Brown *et al.*, 2012), and appear to represent autotetraploid forms of the latter taxon (Atchison, 1948; Coulaud *et al.*, 1995; Brown *et al.*,

2012). However, how *A. heterophylla* and *A. koa* arrived at two opposite sides of the planet remains unresolved. Some have argued that *A. heterophylla* on Réunion Island originated from the relatively older Hawaiian archipelago and thus from *A. koa* (Cheke & Hume, 2008). Others have favoured the explanation of two independent introductions to the Hawaiian Islands and Réunion Island (Carlquist, 1980; Geesink *et al.*, 1990; Brown *et al.*, 2012). Irrespective of the number of introductions, how exactly these two taxa made it to their respective island destinations remains a puzzle. Both species have small thin-walled seeds that are not obviously well adapted for prolonged survival in seawater, and therefore for dispersal in ocean currents. In fact, seeds of *A. koa* are unable to float in seawater and unopened mature pods will only float for 1–2 d (Carlquist, 1966). Also, both species historically grow at altitudes above 450 m – that is, nowhere near the sea (Cheke & Hume, 2008; p. 53). Carlquist (1966) also suggested that koa tree branches carrying mature seed pods may be able to drift in ocean currents for prolonged periods of time and argued in favour of oceanic dispersal, whereas others have suggested dispersal by seabirds (Cheke & Hume, 2008; Kull & Rangan, 2008). Brown *et al.* (2012) supported the 'two independent introductions' hypothesis, and suggested that early humans moved *A. heterophylla* and *A. koa* to their current island homes from Asia, which, in turn, raises questions about the status of the species as native components (*sensu* Pyšek *et al.*, 2004) of their respective floras. Species of Australian acacias are among the world's most problematic invasive plants (Richardson & Rejmánek, 2011; Richardson *et al.*, 2011; Kueffer *et al.*, 2013; Rejmánek & Richardson, 2013). Neither *A. heterophylla* nor *A. koa* are listed among the 24 species in this group known to be invasive (Richardson & Rejmánek, 2011; Rejmánek & Richardson, 2013). If Brown *et al.*'s (2012) interpretation of human-mediated dispersal of the species is correct, these two species should be included in this list (*sensu* Pyšek *et al.*, 2004), thereby adding an exciting new angle and raising new questions for consideration regarding the invasion ecology of the group. Brown *et al.*'s (2012) phylogenetic treatment also confirmed that *A. heterophylla* and *A. koa* diverged from the Australian *A. melanoxylon* very recently, suggesting that the striking morphological similarities between these two island species are probably not the result of convergent evolution. These findings also give credence to the idea of a secondary colonization event from one island group to the other. Thus, despite the recent interest in research on the molecular systematics of Australian acacias (Murphy *et al.*, 2010; Brown *et al.*, 2012; Miller *et al.*, 2013), robust explanations for the peculiar geographic distribution of these two iconic island endemics remain elusive.

Here, we test hypotheses on the origin and biogeographic history of *A. heterophylla* and *A. koa* using phylogeographic, cytogenetic and population genetic approaches. We also use measures of niche overlap to quantify niche conservatism between these two taxa and their sister species, *A. melanoxylon*, from Australia. Specifically, we aim to compare population genetic diversity and structure between *A. heterophylla*, *A. koa* and *A. melanoxylon*. Our expectation is that two independent island introductions would have resulted in monophyly for each island lineage and unique



Fig. 1 *Acacia koa* from the Hawaiian Islands (a, b) and *A. heterophylla* from Réunion Island (c, d), showing the strong morphological similarities between these two island endemics. Photographs courtesy of Johannes Le Roux.

allele diversity. However, if *A. heterophylla* in the Mascarene archipelago originated via a secondary introduction from the Hawaiian archipelago, or vice versa, we would expect to find lower and less unique genetic diversity in the recipient archipelago compared with the donor archipelago, and possibly paraphyly. Despite considerable debate on niche conservatism (e.g. see Peterson *et al.*, 1999), it is conceivable that allopatric speciation can lead to conserved niches (Wiens & Graham, 2005). One can therefore assume that a secondary colonization event would also have left a considerable climatic niche overlap in the native regions of *A. heterophylla* and *A. koa*.

Materials and Methods

Study species

Acacia heterophylla Willd is the only acacia species found in the Mascarenes and even in the whole eco-region (South West Indian Ocean; Polhill, 1990), and is curiously restricted to Réunion Island (where it is called 'tamarin des hauts'). *Acacia heterophylla* is found at mid-elevations (1200–2300 m). *Acacia koa* A. Gray (commonly called koa) is endemic to the Hawaiian archipelago and is a dominant forest canopy tree that can grow up to 35 m tall (Whitesell, 1964). Koa is found on six of the eight main Hawaiian Islands and commonly grows at elevations between 500 and 1500 m (Whitesell, 1990). Although seed production is copious, many koa stands regenerate vegetatively through root suckers, a life history trait typical of many species in the genus (Gibson *et al.*, 2011). Morphologic diversity within koa has led to the recognition of several different taxa (Geesink *et al.*, 1990), although studies examining genetic diversity among these support the recognition of only two species, the widespread and diverse *A. koa* and a localized species *A. koaia* (Fredua-Agyeman *et al.*, 2008; Adamski *et al.*, 2012). Although *A. koa* and *A. heterophylla* are morphologically very similar (Vassal, 1969), differences based on the connation of petals, size of pods and seeds, and the structure of compound pinnae exist between these two taxa (Vassal, 1969; Geesink *et al.*, 1990). Both *A. heterophylla* and *A. koa* are highly valued for their wood. *Acacia melanoxylon* R. Br. is a common tree in its native eastern Australian distributional range and occupies a range of habitats, favouring high rainfall areas, especially in low-lying regions (Maslin & McDonald, 2004). The species has been introduced to numerous regions globally for tannin production, and is currently considered invasive in 11 of the 15 regions of the world defined by Richardson & Rejmánek (2011).

Sampling and DNA extraction

Phyllodes of *A. koa* plants were collected from the native range in the Hawaiian Islands or DNA samples were obtained from The Hawaiian Plant DNA Library, housed at the Department of Botany, University of Hawai'i at Manoa (Morden *et al.*, 1996) representing samples from the islands of Hawai'i, Kaua'i, Maui and O'ahu (18 localities). Phyllodes of *A. heterophylla* were collected from throughout the native range distribution in Réunion

Island (eight localities). Phyllode material for Australian *A. melanoxylon* was collected from southern Australia (three localities) in the native range and from one population in its invasive range in South Africa. Locality data for collections are given in Table 1. In total, 88 accessions representing all three species were included.

Collected phyllode material was dried and stored on silica gel until DNA extraction. Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990) with the addition of 0.2 M sodium sulfite to the extraction and wash buffers (Byrne *et al.*, 2001). DNA quality and quantity were measured using a Nanodrop spectrophotometer (Infinite 200 PRO NanoQuant; Tecan Group Ltd, Männedorf, Switzerland), and all DNA extractions were diluted to a final concentration of *c.* 100 ng μL^{-1} .

DNA sequencing

One nuclear (external transcribed spacer, ETS) and two chloroplast (*rpl32-trnL* and *trnL-F*) intergenic spacers (IGS) were amplified for 43 accessions from across our sampling range. The nuclear ETS was amplified using the primers described in Brown *et al.* (2008) and the PCR set-up and conditions described in Le Roux *et al.* (2011). The chloroplast regions were amplified using the primers described in Shaw *et al.* (2007) and the following PCR conditions: each 50- μL reaction contained *c.* 500 ng of genomic DNA, 200 μM of each deoxynucleoside triphosphate (dNTP) (AB gene; supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 25 pmol of each primer, 5 U Taq DNA polymerase (Super-Therm JMR-801; Southern Cross Biotechnologies), 5 μL of 10 \times PCR reaction buffer and 2 mM MgCl_2 . For the *rpl32-trnL* IGS, amplification conditions consisted of initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 60 s, and a final extension of 72°C for 10 min. For the *trnL-F* IGS, amplification conditions consisted of initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 60 s, 55°C for 60 s and 72°C for 90 s, and a final extension of 72°C for 7 min. Amplified DNA fragments were purified using the QIAquick PCR Purification kit (Qiagen, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (forward only) and an automated ABI PRISM 377XL DNA sequencer (PE Applied Biosystems, Foster City, CA, USA).

Phylogenetic analyses and molecular dating

Contiguous sequences were constructed, edited and aligned using BioEdit version 7.0.5.3 (Hall, 1999) for all DNA regions. All edited sequences have been deposited in GenBank (<http://www.ncbi.nlm.nih.gov>, Table 1). We used several acacia species closely related to *A. koa*, *A. heterophylla* and *A. melanoxylon* (Miller *et al.*, 2011) as outgroup taxa. Relationships among cpDNA haplotypes (contiguous *rpl32-trnL* and *trnL-F* IGS sequences) were examined using statistical parsimony to reconstruct a haplotype network generated at the 95% connection limit with TCS v.1.21

Table 1 Locality data for *Acacia heterophylla*, *A. koa* and *A. melanoxylon* accessions used in this study

Sample ID	Site description	Latitude	Longitude	Elevation ¹	GenBank accession numbers ²
<i>Acacia heterophylla</i> – Réunion Island					
Lu1	Bebour	–21.11504	55.56515	1346	KJ782053, KJ782094, KJ782137
Lu2	Bebour	–21.12005	55.56775	1335	-----
Lu5	Bebour	–21.11504	55.56515	1346	KJ782054, KJ782095, KJ782138
Lu7	Bebour	–21.12005	55.56775	1335	-----
Lu8	Bebour	–21.11504	55.56515	1346	KJ782055, KJ782096, KJ782139
Lu9	Caldera rim	–21.19366	55.64494	2216	KJ782056, KJ782097, KJ782140
Lu11	Caldera rim	–21.19366	55.64494	2216	-----
Lu12	Caldera rim	–21.19366	55.64494	2216	-----
Lu13	Caldera rim	–21.19366	55.64494	2216	KJ782057, KJ782098, KJ782141
Lu15	Caldera rim	–21.19366	55.64494	2216	-----
Lu16	Caldera rim/volcano rim	–21.21076	55.60981	1959	-----
Lu17	Caldera rim/volcano rim	–21.21076	55.60981	1959	KJ782058, KJ782099, KJ782142
Lu18	Caldera rim/volcano rim	–21.21076	55.60981	1959	-----
Lu19	Caldera rim/volcano rim	–21.21076	55.60981	1959	KJ782059, KJ782100, KJ782143
Lu20	Cilaos	–21.12523	55.49007	1450	KJ782060, KJ782101, KJ782144
Lu21	Crossing between national road and volcano road	–21.19785	55.57950	1608	-----
Lu22	Crossing between national road and volcano road	–21.19785	55.57950	1608	KJ782061, KJ782102, KJ782145
Lu23	Crossing between national road and volcano road	–21.19785	55.57950	1608	KJ782062, KJ782103, KJ782146
Lu24	Crossing between national road and volcano road	–21.19785	55.57950	1608	-----
Lu25	La Réunion	NA	NA	1552	KJ782063, KJ782104, KJ782147
Lu27	Piton des Neiges – Track GRR1	–21.11382	55.48794	2011	KJ782064, KJ782105, KJ782148
Lu30	Piton des Neiges – Track GRR1	–21.11753	55.48679	1842	KJ782065, KJ782106, KJ782149
Lu32	Piton des Neiges – Track GRR1	–21.12110	55.48465	1534	-----
Lu33	Piton des Neiges – Track GRR1	–21.11382	55.48794	2011	KJ782066, KJ782107, KJ782150
Lu34	Piton des Neiges – Track GRR1	–21.11382	55.48794	2011	-----
Lu36	Piton Hyacinthe	–21.21769	55.53850	1382	KJ782067, KJ782108, KJ782151
Lu38	Piton Hyacinthe	–21.21763	55.53854	1376	KJ782068, KJ782109, KJ782152
Lu39	Piton Hyacinthe	–21.21763	55.53854	1376	-----
Lu40	Piton Hyacinthe	–21.21763	55.53854	1376	KJ782069, KJ782110, KJ782153
Lu43	Piton Hyacinthe	–21.21769	55.53850	1382	-----
<i>Acacia koa</i> – Hawaiian Islands					
AK573	Pohakuloa, Hawai'i	NA	NA	NA	KJ782070, KJ782111, KJ782154
AK997	Kokee State Park, Kaua'i	22.12033	–159.63702	1132	KJ782071, KJ782112, KJ782155
AK1871	Hawai'i Loa Ridge, O'ahu	21.30053	–157.74550	331	-----
AK1888	Kahana Valley, O'ahu	21.52932	–157.89763	74	KJ851724, KJ782113, KJ782156
AK2005	Kipukapuaulu, Hawai'i Volcanoes National Park, Hawai'i	19.44272	–155.30337	1247	KJ782072, KJ782114, KJ782157
AK2007	Kipukapuaulu, Hawai'i Volcanoes National Park, Hawai'i	19.44272	–155.30337	1247	-----
AK2008	Kipukapuaulu, Hawai'i Volcanoes National Park, Hawai'i	19.44272	–155.30337	1247	-----
AK2009	Kipukapuaulu, Hawai'i Volcanoes National Park, Hawai'i	19.44272	–155.30337	1247	-----
AK2021	Laupahoehoe Reserve, Hawai'i	19.92068	–155.24874	903	KJ782073, KJ782115, KJ782158
AK2023	Laupahoehoe Reserve, Hawai'i	19.92068	–155.24874	903	KJ782074, KJ782116, KJ782159
AK2024	Laupahoehoe Reserve, Hawai'i	19.92068	–155.24874	903	-----
AK2025	Laupahoehoe Reserve, Hawai'i	19.92068	–155.24874	903	-----
AK2037	Koaia Tree Sanctuary, Hawai'i	NA	NA	NA	KJ782075, KJ782117, KJ782160
AK2379	Mahana Ridge, west Maui	20.98962	–156.62095	300	KJ782076, KJ782118, KJ782161
AK2395	Makawao Forest Reserve, east Maui	20.83355	–156.26827	877	KJ782077, KJ782119, KJ782162
AK2415	Kapunakea Forest Reserve, west Maui	NA	NA	NA	KJ782078, KJ782120, KJ782163
AK2713	Ho'omolino Nat. Pres., Hawai'i	NA	NA	NA	KJ782079, KJ782121, KJ782164
AK2714	Ho'omolino Nat. Pres., Hawai'i	NA	NA	NA	KJ782080, KJ782122, KJ782165
AK2745	Wa'ahila Ridge, O'ahu	21.32212	–157.78856	458	KJ782081, KJ782123, KJ782166
AK2979	Hanalei, Kaua'i	22.18532	–159.51524	204	KJ782082, KJ782124, KJ782167
AK2980	Hanalei, Kaua'i	22.18532	–159.51524	204	KJ782083, KJ782125, KJ782168
AK2995	Anahola, Kaua'i	22.15739	–159.34450	386	KJ782084, KJ782126, KJ782169
AK2997	Anahola, Kaua'i	22.15739	–159.34450	386	KJ782085, KJ782127, KJ782170
MJK001	Kalawaline Trail, Tantulus Drive, O'ahu	21.33401	–157.81868	432	-----
MJK002	Kalawaline Trail, Tantulus Drive, O'ahu	21.33401	–157.81868	432	-----
MJK003	Kalawaline Trail, Tantulus Drive, O'ahu	21.33401	–157.81868	432	-----
MJK004	Kalawaline Trail, Tantulus Drive, O'ahu	21.33003	–157.81433	481	-----
MJK005	Kalawaline Trail, Tantulus Drive, O'ahu	21.33003	–157.81433	481	-----
MJK007	Kalawaline Trail, Tantulus Drive, O'ahu	21.33003	–157.81433	481	-----

Table 1 (Continued)

Sample ID	Site description	Latitude	Longitude	Elevation ¹	GenBank accession numbers ²
MJK008	Kalawaline Trail, Tantulus Drive, O'ahu	21.33003	−157.81433	481	-----
MJK010	Crater Road, Haleakala Ranch, Maui	20.77682	−156.23368	1906	-----
MJK011	Crater Road, Haleakala Ranch, Maui	20.77682	−156.23368	1906	-----
MJK013	Crater Road, Haleakala Ranch, Maui	20.77682	−156.23368	1906	-----
MJK019	Waikamoi Mauka, The Nature Conservancy, Maui	20.83105	−156.18265	601	-----
MJK020	Waikamoi Mauka, The Nature Conservancy, Maui	20.83105	−156.18265	601	-----
MJK021	Waikamoi Mauka, The Nature Conservancy, Maui	20.83105	−156.18265	601	-----
MJK022	Waikamoi Mauka, The Nature Conservancy, Maui	20.83105	−156.18265	601	-----
MJK023	Waikamoi Mauka, The Nature Conservancy, Maui	20.83105	−156.18265	601	-----
MJK029	Waikamoi Makai, Haleakala Ranch, Maui	20.79411	−156.25099	1576	-----
MJK030	Waikamoi Makai, Haleakala Ranch, Maui	20.79411	−156.25099	1576	-----
MJK031	Waikamoi Makai, Haleakala Ranch, Maui	20.79411	−156.25099	1576	-----
MJK032	Waikamoi Makai, Haleakala Ranch, Maui	20.79411	−156.25099	1576	-----
MJK033	Waikamoi Makai, Haleakala Ranch, Maui	20.79411	−156.25099	1576	-----
<i>Acacia melanoxylon</i> – Australia and South Africa					
CM25	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM27	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM28	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM29	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM31	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	KJ782086, KJ782128, KJ782171
CM32	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM36	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	KJ782087, KJ782129, KJ782172
CM37	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM41	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM42	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM43	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM45	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM46	Farmer's Choice Estate, Western Cape, South Africa	−33.96969	23.43934	223	-----
CM49	Mount Compass, South Australia	−35.40585	138.59882	311	KJ782088, KJ782130, KJ782173
CM50	Mount Compass, South Australia	−35.40585	138.59882	311	KJ782089, KJ782131, KJ782174
CM53	Mount Compass, South Australia	−35.40585	138.59882	311	KJ782090, KJ782132, KJ782175
CM54	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM57	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM58	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM59	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM60	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM61	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM62	Mount Compass, South Australia	−35.40585	138.59882	311	KJ782091, KJ782133, KJ782176
CM63	Mount Compass, South Australia	−35.40585	138.59882	311	-----
CM65	Kuitpo forest, South Australia	−35.20750	138.70000	303	KJ782092, KJ782134, KJ782177
CM66	Kuitpo forest, South Australia	−35.20750	138.70000	303	-----
CM68	Kuitpo forest, South Australia	−35.20750	138.70000	303	-----
CM69	Off South Gippsland Highway, South Australia	−38.44788	145.91312	84	KJ851725, KJ782135, KJ782178
CM70	Off South Gippsland Highway, South Australia	−38.44788	145.91312	84	-----
CM72	Off South Gippsland Highway, South Australia	−38.44788	145.91312	84	KJ782093, KJ782136, KJ782179

¹Metres above sea-level.²Only taxa with GenBank accession numbers were included in the phylogenetic and network analyses, whereas all taxa were included in the amplified fragment length polymorphism (AFLP) analysis. Order of GenBank accession numbers: *rpl32-trnL*, *trnL-F* and ETS. NA, not available.

(Clement *et al.*, 2000). Gaps were discarded in the network analysis.

The full dataset of all three gene regions (ETS, *rpl32-trnL* and *trnL-F*) was analysed using Bayesian search criteria with parameter estimates obtained from the program jModelTest v2.1.3 (Darriba *et al.*, 2012) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). MrBayes was run for 1000 000 generations and trees were sampled every 1000 generations. The nodal support of the retrieved tree topology was determined as posterior probabilities in MrBayes. A likelihood test for equal rates of evolution of

our data was conducted in MEGA v.5.1 (Tamura *et al.*, 2011). The latter rejected equal rates of evolution and, for the dating analysis, we therefore used a relaxed molecular clock approach as implemented in BEAST v1.7.5 (Drummond *et al.*, 2002, 2012). The input data were compiled using BEAUti v1.5.3 with the tree priors set as follows: age for the monophyletic clade including all Hawaiian koa of 5.1 million yr (corresponding to the age of the oldest current Hawaiian high island, Kaua'i; McDougall, 1979) with lognormal prior distribution and the Yule process of speciation. The HKY+G model was specified as the best-fitting

evolutionary model based on the Akaike Information Criterion in jModelTest v2.1.3 (Darriba *et al.*, 2012). Five separate runs were performed in BEAST with 10 million generations each, sampling every 1000th generation. In order to guarantee that the Markov chain Monte Carlo (MCMC) chain had run long enough to obtain a valid approximation of the parameters, individual log files were analysed with Tracer v1.5 (Rambaut & Drummond, 2009) to assess convergence and to confirm that the combined effective sample sizes for all parameters were larger than 200. All resulting trees were combined using LogCombiner v1.5.3 (Drummond & Rambaut, 2007b), with a burn-in of 25%. A single maximum credibility tree was then produced using TreeAnnotator v1.7.5 (Drummond *et al.*, 2012) and visualized using Figtree v1.4 (Rambaut, 2012). Lastly, we also computed a matrix of pairwise genetic distances between *A. koa* and *A. heterophylla* accessions based on all three gene regions using DNADist in BioEdit (Hall, 1999).

Amplified fragment length polymorphism (AFLP) analyses

PCR amplification of AFLP fragments was performed for all sampled accessions using the 'universal' protocol described by Blignaut *et al.* (2013). Briefly, following digestion and pre-selective PCR, selective PCR amplification with two different fluorescently labelled *EcoRI* primers (*EcoRI*-AAT and *EcoRI*-CAT) was performed.

Automated fragment size calling and scoring were performed using Genemarker version 2.2.0 (SoftGenetics LLC, State College, PA, USA). The presence or absence of all fragments was manually confirmed to avoid false absences as a result of intensity differences between samples. All procedures were repeated on 10% of all samples in order to assess repeatability.

AFLP fragments were scored as either present (1) or absent (0) to create a binary matrix. PopGene v1.32 (Yeh *et al.*, 1997) was used to estimate genetic diversity within populations (in our case species) as the number of effective alleles (N_E), Nei's (1973) gene diversity (h), Shannon's information index (I) (Shannon & Weaver, 1949) and the percentage of polymorphic loci (PP). An analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992), as implemented in GenAlEx v6.41 (Peakall & Smouse, 2006), was used to estimate the distribution of population genetic variation within and between species as Φ_{PT} (analogous to Wright's fixation index or F_{ST}).

Genetic structure

To assess genetic structure between *A. koa*, *A. heterophylla* and *A. melanoxylon*, Bayesian assignment tests based on the AFLP data were performed to assign individual genotypes probabilistically to populations using STRUCTURE v2.2 (Falush *et al.*, 2007). For the STRUCTURE analysis, simulations were run with between one and five populations or genetic clusters (i.e. $K=1-5$) using the option of taking population affiliation into account and allowing admixture. Ten runs of one million iterations, followed by a burn-in period of 100 000 for each K value, were performed. In addition, LnProb values obtained from the

STRUCTURE analysis were used to calculate ΔK (Evanno *et al.*, 2005) to estimate the optimum number of genetic clusters.

Flow cytometry analysis

We determined the relative genome sizes of silica gel-dried specimens by flow cytometry to confirm previous reports of polyploidy in *A. koa* and *A. heterophylla*. To isolate nuclei, dried leaf material from each specimen was chopped together with an appropriate volume of the internal standard (*Pisum sativum*, 2C = 9.09 pg) in ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The resulting suspension was filtered through a 42- μ m nylon mesh and left at room temperature for 10 min. Isolated nuclei were then stained with 1 ml of Otto II buffer (0.4 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) supplemented with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 4 $\mu\text{g ml}^{-1}$ and β -mercaptoethanol (2 $\mu\text{l ml}^{-1}$). After a few minutes, the relative fluorescence intensity of particles was recorded using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp as the source of UV excitation light. Flow histograms were evaluated using Partec FloMax software ver. 2.4d with the fluorescence intensity of *P. sativum* set to unity and fluorescence values of acacias expressed as peak ratios against this reference standard.

Ecological niche overlap

We quantified differences in ecological niches between *A. melanoxylon*, *A. koa* and *A. heterophylla* based on niche overlap. We expect closely related taxa to share similar ecological niches and therefore to be congruent with the phylogenetic relationships retrieved among these taxa. Although there has been considerable debate on the quantification of ecological niche space (see Peterson *et al.*, 1999), recent studies have shown that comparisons between taxa have been limited by methodological shortcomings, and therefore more robust methods have been proposed recently (Warren *et al.*, 2008; Broennimann *et al.*, 2012). We measured niche equivalency (whether niches of two taxa in two geographical ranges are equivalent) and niche similarity (whether the niche occupied in one range is more similar to that occupied in another range than would be expected by chance) between the pairs *A. melanoxylon/A. koa* and *A. koa/A. heterophylla*. These comparisons follow the proposed sequence of colonization of *A. melanoxylon* from Australia to the Hawaiian Islands and of *A. koa* from the Hawaiian Islands to Réunion Island based on our genetic data (see the Results section). Measures of niche overlap are based on a robust framework considering species density and the availability of climatic data in both ranges (i.e. analogous climates, Broennimann *et al.*, 2012). The niche equivalency test determines whether the niche overlap is constant when randomly reallocating the occurrences of both species among the two ranges. This process is repeated 100 times to create a null distribution. The niche similarity tests randomly shift the entire observed density of occurrences in one range and calculate the overlap of the simulated niche with the observed niche in the other range. This process is also repeated 100 times to create a null distribution.

Several ordination techniques can be applied to calculate the amount of niche overlap D , which ranges from 0 (no overlap) to 1 (complete overlap). Here, we used the first two axes of a principal component analysis calibrated on the entire environmental space of the two study areas (Australia/Hawaiian Islands and Hawaiian Islands/Réunion Island). The methods have been described in detail in Broennimann *et al.* (2012). A dataset of 256 geo-referenced localities of *A. melanoxylon* (source: Australian Virtual Herbarium, <http://avh.chah.org.au/>), 99 of *A. koa* and 70 of *A. heterophylla* was used (sources: this study, Bishop Museum, Honolulu, Hawai'i and Global Biodiversity Information Facility (www.gbif.org)). Localities were selected to encompass the full known range of each species in undisturbed habitats. Environmental space was defined by six variables representing the current climate (mean annual precipitation, mean annual temperature, maximum temperature of the warmest month, minimum temperature of the coldest month, precipitation of the wettest quarter and precipitation of the driest quarter) extracted from the WorldClim database (<http://www.worldclim.org>) at a 30 arc second resolution. All analyses were performed in R (R Development Core Team, 2010), based on the script by Broennimann *et al.* (2012).

Results

Network analysis, phylogeny and molecular dating

The aligned *trnL-F* matrix contained 996 characters, the *rpl32-trnL* matrix 586 characters and the ETS matrix 432 characters. All DNA sequences have been deposited in GenBank (Table 1). The alignment matrix of all sequences combined into single contigs required 17 gaps (indels), ranging from one to 16 characters in size.

A parsimony network analysis indicated that all *A. heterophylla* samples from Réunion Island shared a single cpDNA haplotype which was closely related (a single mutational step) to haplotypes of *A. koa* from the Hawaiian Islands (Fig. 2). Overall, the single *A. heterophylla* haplotype fell within the Hawaiian haplotype group. Hawaiian accessions of *A. koa* were also notably more diverse, comprising nine haplotypes. These haplotypes were very divergent (15 mutational steps) from *A. melanoxylon* haplotypes (Fig. 2).

Our phylogeny (cpDNA and nDNA combined) supported our network analysis (cpDNA only), indicating that *A. heterophylla* rendered *A. koa* paraphyletic (Figs 3,4). Overall, the retrieved tree topology was highly supported (posterior probabilities). Constraining the node of the clade including both *A. koa* and *A. heterophylla* at 5.1 million yr ago (Ma; oldest age of the current high island of Kaua'i, McDougall, 1979) indicated that the *A. heterophylla* clade diverged from *A. koa* c. 1.4 Ma (861 ka to 2.04 Ma).

AFLP population genetic diversity and structure

Forty-five loci were reliably scored for every individual from the two AFLP primers used. Re-analysis (i.e. re-amplification, re-genotyping and re-scoring) of 10% of the total sample sizes

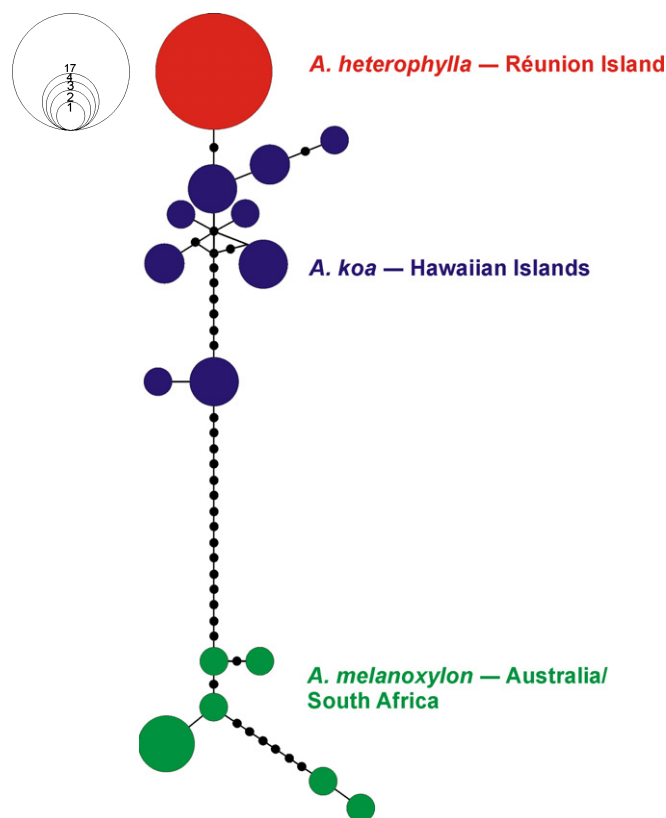


Fig. 2 Parsimony haplotype network based on the chloroplast *rpl32-trnL* and *trnL-F* intergenic spacer (IGS) regions. Each circle represents a single haplotype and each haplotype's frequency is related to the surface area of each circle (see inset for guide). Black dots represent mutation steps separating connected haplotypes.

revealed high repeatability with an estimated error rate of 1.2%. With regard to genetic diversity, the effective number of alleles (N_E), Nei's gene diversity (h), Shannon's information index (I) and the percentage of polymorphic loci (PP) were highly similar for all three species (Table 2).

Bayesian assignment tests identified two genetic clusters ($K=2$), the first corresponding to *A. koa* and *A. heterophylla*, and the second to *A. melanoxylon* (Fig. 5). Furthermore, higher genetic differentiation was observed between Australian *A. melanoxylon* and the two island endemics (*A. koa*, $\Phi_{PT}=0.318$; *A. heterophylla*, $\Phi_{PT}=0.377$) than between the two island endemics ($\Phi_{PT}=0.207$).

Genome size variation

Our flow cytometry results confirmed that *A. koa* and *A. heterophylla* most likely represent tetraploid forms (relative fluorescence intensities of 0.393 ± 0.002 and 0.405 ± 0.002 , respectively, as against *Pisum sativum* with a unit value) of *A. melanoxylon* (relative fluorescence intensity of 0.203 ± 0.001).

Ecological niche overlap

We found significant differences in niche overlap between Australian *A. melanoxylon* and *A. koa* from the Hawaiian Islands,

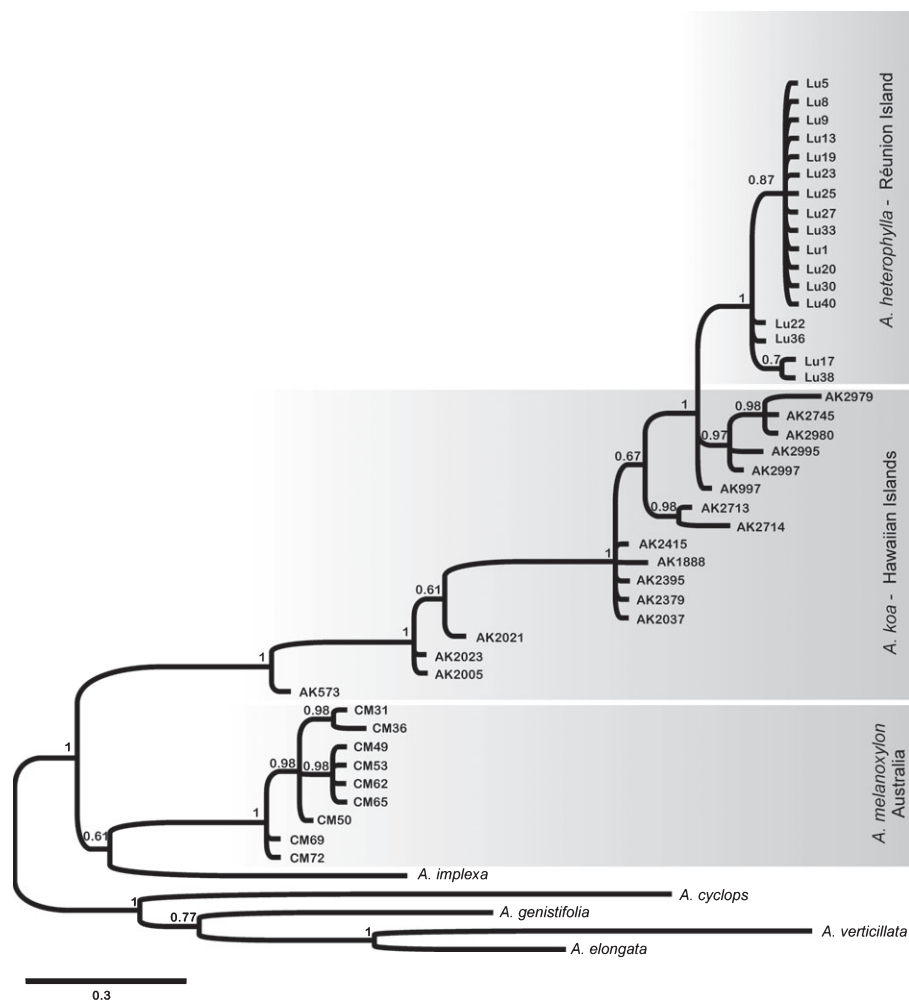


Fig. 3 Bayesian phylogeny based on all gene regions (ETS, *rpl32-trnL* and *trnL-F*), illustrating phylogenetic relationships among *Acacia heterophylla*, *A. koa* and *A. melanoxylon*. Nodal support (posterior probabilities) is indicated above the branches.

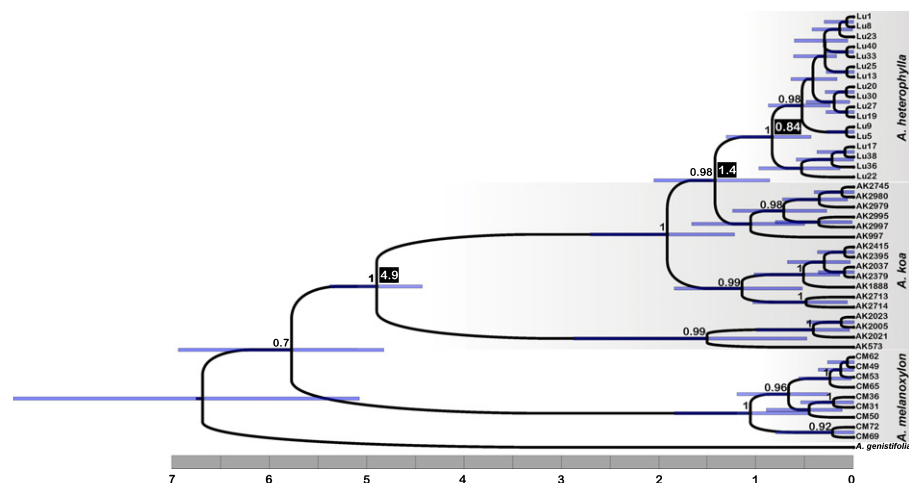


Fig. 4 BEAST chronogram showing the relationships between *Acacia heterophylla*, *A. koa* and *A. melanoxylon* based on combined data of ETS, *rpl32-trnL* and *trnL-F* gene regions. Blue bars represent 95% highest probability density (HPD) intervals. Number on the scale bar shows millions of years before present. Topology support is given as posterior probabilities above the branches, whereas important estimated dates of diversification (million yr ago, Ma) are indicated in black boxes next to the relevant nodes.

but less difference between the two island endemics, *A. koa* and *A. heterophylla*. We rejected the hypothesis of niche equivalency between *A. melanoxylon* and *A. koa* ($D=0.144$, $P<0.01$) and between *A. koa* and *A. heterophylla* ($D=0.121$, $P<0.01$) (Fig. 6). However, ecological niches between the two archipelago endemic species were more similar than expected by chance ($P<0.05$) (Fig. 6).

Discussion

Our genetic results not only suggest that *A. koa* from the Hawaiian Islands and *A. heterophylla* from Réunion Island represent extremely closely related genetic lineages, but also, more likely from a phylogenetic point of view, the same species (*sensu* Donoghue, 1985). The results also show that it is highly unlikely

Table 2 Summary statistics for amplified fragment length polymorphism (AFLP) genetic data for *Acacia koa*, *A. heterophylla* and *A. melanoxylon* based on 45 loci

<i>Acacia</i> species	N_a ¹	N_E ²	h ³	I ⁴	pp ⁵
<i>A. heterophylla</i>	1.42	1.24	0.15	0.22	42.2
<i>A. koa</i>	1.47	1.23	0.14	0.21	46.7
<i>A. melanoxylon</i>	1.49	1.20	0.13	0.20	48.9

¹Observed number of alleles.

²Effective number of alleles (Kimura and Crow, 1964).

³Nei's (1973) gene diversity.

⁴Shannon's information index (Lewontin, 1972).

⁵Percentage of polymorphic loci.

that these two island endemics arose from two independent long-distance dispersal events from Australia, but rather that secondary dispersal occurred between these two isolated insular landmasses. This is evident from the strong monophyly retrieved for these two island endemics in relation to *A. melanoxylon* and *A. heterophylla*, rendering *A. koa* paraphyletic (Figs 3,4). More specifically, given the relatively higher phylogenetic diversity found in the Hawaiian Islands and the tree topology retrieved here (Figs 2,3), the most parsimonious explanation is that *A. heterophylla* in Réunion Island originated from an extreme long-distance dispersal event from the Hawaiian Islands. Our dated phylogeny suggests that this may have occurred fairly recently, *c.* 1.4 Ma, and that diversification of *A. heterophylla* lineages within Réunion occurred as recently as 840 ka. Colonization of Réunion Island may have even occurred earlier, that is

< 1.4 Ma, as our dated phylogeny was calibrated using the oldest possible age of the current high islands of the Hawaiian archipelago, thus reflecting the oldest possible divergence between *A. koa* and *A. heterophylla*.

Our results show that previous suggestions of human-aided dispersal events among these two island endemics, occurring from Asia (Brown *et al.*, 2012), are clearly incorrect. Furthermore, numerous attributes strongly suggest historical natural long-distance dispersal of the ancestral species of *A. koa* from Australia to Hawai'i rather than human-assisted dispersal. For example, the genetic diversity and structure found in and among *koa* populations in Hawai'i (Fredua-Agyeman *et al.*, 2008; Adamski *et al.*, 2012) far exceed that which would be expected to have developed following human introduction < 1500 yr ago. Genetic differentiation among *koa* populations is consistent with that which has been found for other Hawaiian endemic species with multi-island distributions, such as the widespread *Metrosideros polymorpha* (Myrtaceae; Wright *et al.*, 2001; Harbaugh *et al.*, 2009) and the endangered *Colubrina oppositifolia* (Rhamnaceae; Kwon & Morden, 2002). Ecological attributes also include evidence from interactions among native Hawaiian species, which was also the rationale for calibrating our phylogeny using the oldest possible geological age of current high Hawaiian Islands. For example, gut content analysis of Hawaiian *koa* finches from the genus *Rhodacanthis*, that went extinct in the late 1800s, show *koa* seeds to have been the primary food source for these birds, to which they were well adapted (Munro, 1944; James & Price, 2008). In line with this, historical pollen records support the dominance of *A. koa* in Hawaiian landscapes long

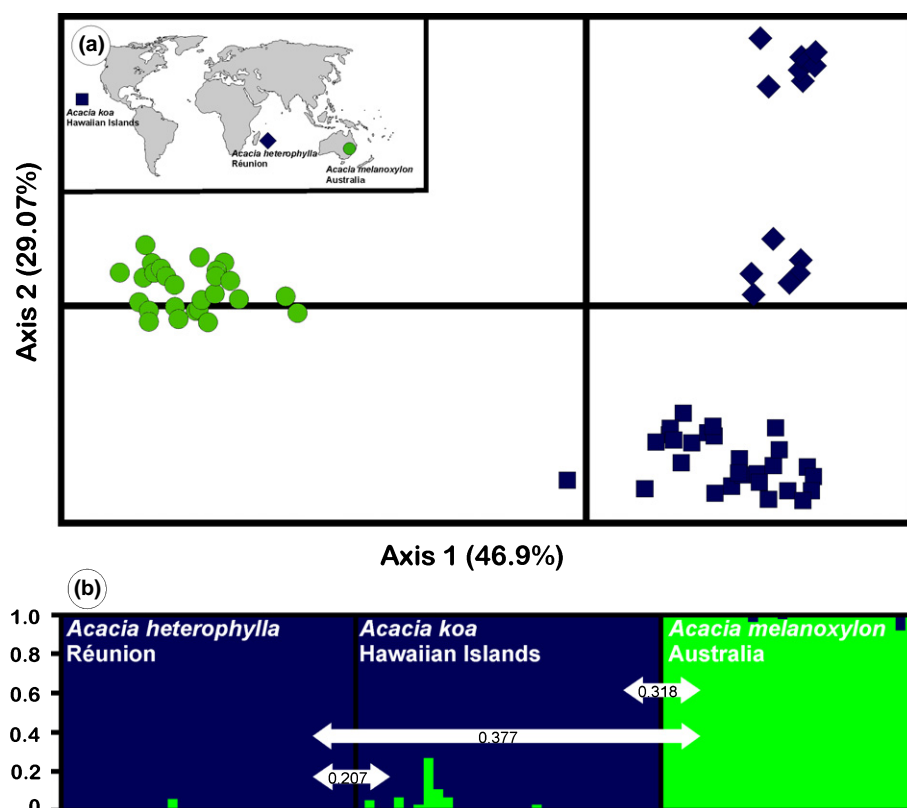


Fig. 5 Population genetic structure based on amplified fragment length polymorphism (AFLP) analysis of *Acacia koa*, *A. heterophylla* and *A. melanoxylon*. (a) Plot of the first two axes of a principal component analysis (PCoA) showing genetic differentiation based on pairwise Φ_{ST} values for all three taxa. The inset map indicates the global regions from which these populations originated. (b) Bayesian assignments of *A. koa*, *A. heterophylla* and *A. melanoxylon* in STRUCTURE. The vertical axes of all bar plots illustrate the proportional assignment of individual genomes to the inferred number of genetic groups/clusters. Population (species) level Φ_{ST} values are indicated by arrows.

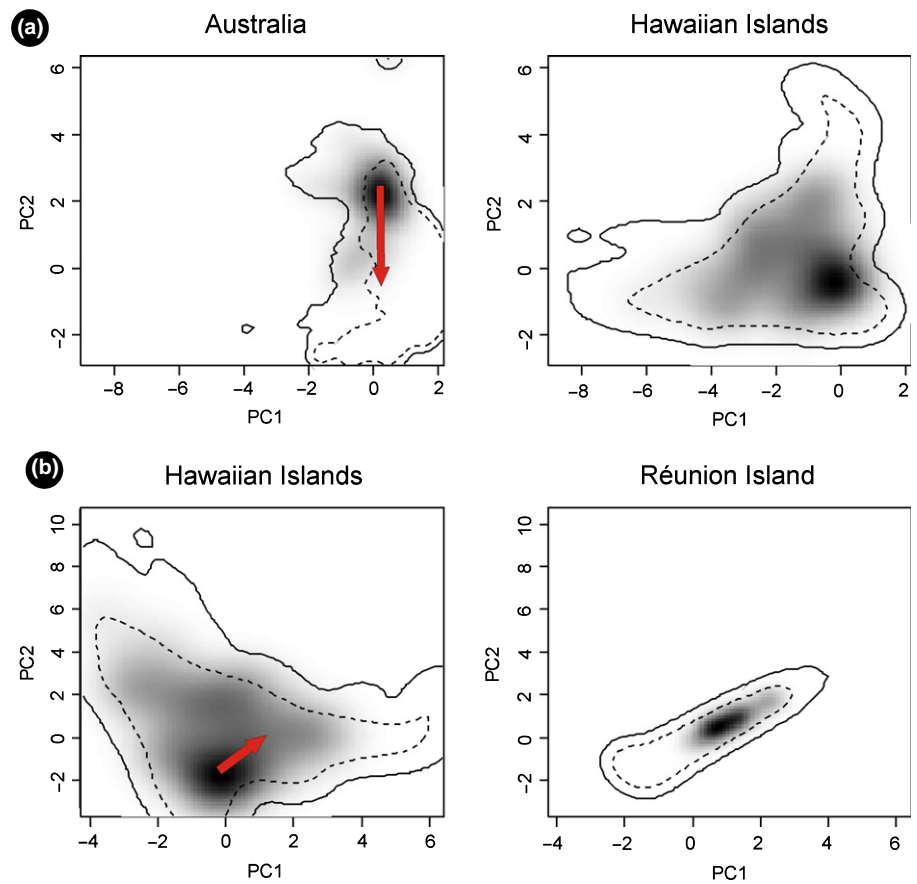


Fig. 6 Changes in ecological niche represented by principal component analyses based on six climatic variables between (a) *Acacia koa* and *A. melanoxyton* and (b) *A. heterophylla* and *A. koa*. Niche equivalency was rejected for (a) and (b), but niche similarity was confirmed for (b) only. Grey shading shows the density of species occurrence; the solid and dashed contour lines represent 100% and 50% of the available environment, respectively. The arrow indicates the direction in which the centre of the niche has shifted.

before the arrival of humans (Hotchkiss & Juvik, 1999), and many endemic Hawaiian insects appear to be host specific on *A. koa* (Gagne, 1979). It is therefore safe to conclude that koa was present on the Hawaiian Islands well before the arrival of humans. However, despite the pre-human presence of koa in the Hawaiian Islands, its colonization of the archipelago's current high islands must have been fairly recent in terms of the geological timescale. It is generally accepted that most of the flora on the current high islands of the Hawaiian archipelago, especially taxa restricted to higher elevations, resulted from colonization and diversification within the past *c.* 5 million yr (Price & Clague, 2002; but see Keeley & Funk, 2011 for notable exceptions). In the case of *A. koa*, certain life history traits suggest a recent colonization event. For example, koa trees have functional extrafloral nectaries, an adaptation to ant mutualisms, despite there being no native ants on the Hawaiian Islands (Keeler, 1985). We could not estimate an approximate age for the colonization of *A. koa* in the Hawaiian Islands, but used the oldest possible age (Kaua'i; McDougall, 1979) to estimate the most conservative timing of a secondary colonization event to Réunion Island. However, *A. koa* may have also been present for much longer, that is, being previously present on the older (now submerged) Hawaiian Islands, followed by colonization of the younger current high islands within the past *c.* 5 million yr. However, our conservative dating approach estimated the split between *A. koa* and *A. melanoxyton* to be *c.* 6 Ma, making this an unlikely colonization scenario.

Our dating analyses confirmed a very recent colonization of Réunion Island by *A. koa*, estimated at ≤ 1.4 Ma. This was confirmed by the genetic distance between *A. koa* and *A. heterophylla*, suggesting very low divergence. For example, the genetic distance between *A. koa* and *A. heterophylla* was far lower (0.003) than known genetic distances between species within the genus *Acacia* and even among subspecies (Thompson *et al.*, 2012). Furthermore, our AFLP analysis indicated a very close genetic relationship between these two species compared with the closely related sister species, *A. melanoxyton* (Fig. 4). Our genetic results were supported by our niche overlap results, indicating that the ecological niche between *A. melanoxyton* and *A. koa* has diverged significantly, but that some elements of niche conservatism (as shown by a niche similarity test) still persist between *A. heterophylla* and *A. koa*, but not *A. melanoxyton* (Fig. 6). These patterns might reflect the effects of polyploidization on ecological tolerance and colonization ability (*sensu* te Beest *et al.*, 2012), leading to similarities between the autotetraploid taxa *A. heterophylla* and *A. koa*, but not between *A. koa* and the diploid *A. melanoxyton*.

Irrespective of their current taxonomic status, that is, whether or not *A. koa* and *A. heterophylla* represent a single taxon, the question remains how these two island endemics reached two small landmasses on opposite sides of the planet. Several authors have argued that gadfly petrels, such as the endemic Barau's Petrel (*Pterodroma baraui*) from Réunion Island, may have transported seeds from the Hawaiian Islands. In Réunion, these birds

dig burrows at elevations that coincide with the distributions of *A. heterophylla* (Cheke & Hume, 2008). Similarly, some bird species in the Hawaiian Islands, including petrels, nest at altitudes overlapping with the distribution of koa (e.g. VanZandt *et al.*, 2014). Plant seeds have been found in the gut contents of petrels (Peter Ryan, The Percy Fitzpatrick Institute of African Ornithology, University of Cape Town, South Africa, pers. comm.). Ingested seeds may be carried for vast distances, as plastic pellets are known to be retained in petrel's ventriculi for weeks (Ryan & Jackson, 1987) and probably months (Ryan, 1988). Seeds (and other materials) may also attach to the feathers of birds or within mud stuck to the legs of birds (Blattner, 2006). It is therefore possible that 'lost petrels from Hawai'i' . . . strayed into the wrong ocean, finding there a new mountainous island at the right latitude' (Cheke & Hume, 2008, pp. 53–54), delivering seed/s of what would later become the endemic 'tamarin des hauts'. Although the molecular data presented here confirm that *A. heterophylla* colonized Réunion Island following dispersal from the Hawaiian Islands, the vector of dispersal and details of the colonization event remain speculative.

The colonization of the Hawaiian Islands by plants of geographically diverse and distant origins, including Australia, has been well documented (Keeley & Funk, 2011). The establishment of *A. koa* as a result of long-distance dispersal is also widely accepted (Fosberg, 1948). Although a link between the Hawaiian Islands and Réunion is hard to conceive, direct African/Mascarene origins have been inferred for some endemic Hawaiian genera, such as *Hesperomannia* (Kim *et al.*, 1998) and *Kokia* (Seelanan *et al.*, 1997), indicating that such extreme long-distance dispersal events, albeit in the opposite direction, have played a role in shaping island biotas. Our results show how extreme long-distance dispersal can produce enigmatic biogeographic patterns and unique patterns of endemism. Although oceanic barriers might be less important in the distribution of some insular taxa, e.g. spore-forming bryophytes (Patiño *et al.*, 2013), our findings indicate that the same might be true even when conventional wisdom predicts otherwise. The biogeographic history of koa and 'tamarin des hauts' on their widely separated island homes offers exciting opportunities for comparative studies to gain new insights into the diversification and evolution of insular floras.

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