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2 **Contribution of transition and stabilization processes to**  
3 **speciation is a function of the ancestral trait state and selective**  
4 **environment in *Hakea***

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9 **ABSTRACT**

10 Currently the origin and trajectories of novel traits are emphasised in evolutionary studies, the  
11 role of stabilization is neglected, and interpretations are often *post hoc* rather than as  
12 hypothesised responses to stated agents of selection. Here we evaluated the impact of  
13 changing environmental conditions on trait evolution and stabilization and their relative  
14 contribution to diversification in a prominent Australian genus, *Hakea* (Proteaceae). We  
15 assembled a time-based phylogeny for *Hakea*, reconstructed its ancestral traits for six  
16 attributes and determined their evolutionary trajectories in response to the advent or  
17 increasing presence of fire, seasonality, aridity, nectar-feeding birds and (in)vertebrate  
18 herbivores/granivores. The ancestral *Hakea* arose 18 million years ago (Ma) and was broad-  
19 leaved, non-spinescent, insect-pollinated, had medium-sized, serotinous fruits and resprouted  
20 after fire. Of the 190 diversification events that yielded the 82 extant species analysed, 8–50%  
21 involved evolution, stabilization or re-evolution (reversal) of individual novel traits. Needle  
22 leaves appeared 14 Ma and increased through the Neogene/Quaternary coinciding with  
23 intensifying seasonality and aridity. Spinescence arose 12 Ma consistent with the advent of

24 vertebrate herbivores. Bird-pollination appeared 14 Ma in response to advent of the  
25 Meliphagidae in the early Miocene. Small and large woody fruits evolved from 12 Ma as  
26 alternative defenses against granivory. Fire-caused death evolved 14 Ma, accounting for 50%  
27 of subsequent events, as fire became less stochastic. Loss of serotiny began in the late  
28 Miocene as non-fireprone habitats became available but only contributed 8% of events.  
29 Innovation and subsequent stabilization of functional traits promoted the overall species  
30 diversification rate in *Hakea* by 15 times such that only three species now retain the ancestral  
31 phenotype. Our approach holds great promise for understanding the processes responsible for  
32 speciation of organisms when the ancestral condition can be identified and the likely selective  
33 agents are understood.

34 **Key words:** Ancestral state reconstruction; drought, fire; fruit size, granivore/herbivore,  
35 *Hakea*; insect/bird pollination; leaf shape, Miocene, serotiny, spinescence, resprouting,  
36 species diversification, trait proliferation/transition/stabilization

37

## 38 INTRODUCTION

39 Studies that capture patterns of speciation associated with changes in environmental  
40 conditions provide compelling support for the key role of functional trait shifts in the process  
41 of evolution by natural selection (Jetz *et al.*, 2012). Natural selection can induce the evolution  
42 of novel traits whose fitness exceeds that of the incumbent trait (directional selection) or  
43 perpetuation of the current trait whose fitness exceeds that of a former or alternative trait  
44 (stabilizing selection) (Lemey *et al.*, 2009). Phylogenetic methods have been developed to  
45 investigate a wide range of questions regarding species evolution, including the inference of  
46 ancestral traits (He *et al.*, 2011, 2012; Crisp *et al.*, 2011) and to address the relationship  
47 between traits and rates of speciation (Litsios *et al.*, 2014). While currently the origin and

48 evolutionary trajectories of novel traits are emphasised the role of stabilization has been  
49 neglected and interpretations have often been *post hoc* rather than as hypothesised responses  
50 to stated agents of natural selection. This is partly because of ignorance of the advent or  
51 strength of the postulated selective agents. Significant questions remain: To what extent do  
52 directional and stabilizing process contribute to trait proliferation? Do their contributions vary  
53 between attributes, traits and/or over geological time? Is the proliferation of a trait at the  
54 expense of its alternative traits? Can patterns of directional and stabilizing selection over time  
55 be interpreted in terms of the advent or changes in the intensity of particular agents of  
56 selection?

### 57 *Theory and concepts*

58 Each alternative state of a species attribute is here termed a trait. Increase in occurrence of a  
59 given trait through a phylogeny is defined as trait proliferation (He *et al.*, 2011, Lamont *et al.*,  
60 2013). The fraction of total diversification events that result in the presence of that trait is the  
61 trait proliferation rate (this may also be given on an absolute basis per unit time, as for species  
62 diversification). Trait proliferation results from two evolutionary processes: transition – a new  
63 trait arises during the event, and stabilization – the trait is conserved during the event.

64 Transition rate (TR) is the fraction of events in which the trait arises relative to the maximum  
65 number in which that trait could occur, while stabilization rate (SR) is the fraction of events  
66 in which the trait is retained relative to the maximum number in which that trait could occur.  
67 Thus, the (net) trait proliferation rate (PR) = TR + SR. Generally, for a pair of opposing traits,  
68 1 and 2, evolving in a clade with a total of  $n/2$  nodes and thus  $n$  diversification events for the  
69 period of interest,  $PR_1 = TR_{2/1} + SR_{1/1}$ ,  $TR_{2/1} = \Sigma(2 \rightarrow 1 \text{ events})/n$ , and  $SR_{1/1} = \Sigma(1 \rightarrow 1 \text{ events})/n$ .  
70 Trait reversals are successive transitions that return the phenotype to the previous trait state in  
71 the lineage, as an inverse function of the stability through time of the selective pressure for

72 that trait. The concepts of rates of diversification, proliferation, stabilization and transition,  
73 and reversals are illustrated with a concrete example in Fig. 1.

74 A new agent of directional selection usually operates in a different spatial or temporal  
75 dimension than the existing agents and becomes a supplementary force that initially retards  
76 speciation and then promotes it once an adapted genotype has evolved followed by rampant  
77 speciation into the “vacant niche” now available as the new trait proliferates (Lamont *et al.*,  
78 2013). The premise here is that the more habitats (niches) a given area can be divided into,  
79 the greater the opportunities for novel genotypes to arise. Accepting that new, alternative  
80 traits supplement rather than replace ancestral traits as options, the contribution of novel traits  
81 to speciation can be calculated as the inverse of the percentage contribution of the ancestral  
82 trait to all subsequent diversification events (Y): speciation promotional rate (SPR) = 100/Y.  
83 Thus, the smaller Y, the greater the contribution of novel traits to the subsequent  
84 diversification events.

#### 85 *Evolution and adaptations of Hakea*

86 Nutrient-poor, fire-prone, Mediterranean-type regions with a prolonged hot, dry season and  
87 exposed to intensive pressure from pollinators, herbivores and granivores are characterised by  
88 high species richness and endemism (Cowling *et al.*, 1996) and should provide suitable  
89 scenarios to examine these issues. *Hakea* is a shrub genus of over 150 species, spread  
90 throughout Australia but best represented in mediterranean southwestern Australia, and  
91 renowned for its great variation in leaf and fruit morphology, pollinators, climate and fire  
92 tolerances and susceptibility to herbivores and granivores (Groom and Lamont, 1996a, 1997,  
93 2015; Lamont *et al.*, 2015, 2016; Hanley *et al.*, 2009, Rafferty *et al.*, 2010).

94 *Hakea* is highly sclerophyllous with needle-leaved and broad-leaved species. Needle leaves  
95 are twice as thick as broad leaves implying that they have had greater exposure to drought

96 and heat (Lamont *et al.*, 2015). Previous molecular analysis indicates that *Hakea* originated in  
97 the early Miocene directly from non-fireprone, rainforest ancestors (Sauquet *et al.*, 2009;  
98 Lamont and He, 2012). *Hakea* most probably originated in the sclerophyll shrublands of  
99 southwestern Australia where it continued to diversify strongly until the present (Lamont *et*  
100 *al.*, 2016). From the mid-Miocene, it gradually speciated and migrated onto recently-exposed,  
101 rocky substrates, sclerophyll forests and woodlands, and across the drier centre of Australia to  
102 the moister margins. Thus, we hypothesise that the ancestral *Hakea* leaves were broad,  
103 reflecting their mesic heritage, that they were retained (or re-evolved) in temperate  
104 environments, but that needle leaves arose in the mid-Miocene and proliferated strongly  
105 through the late-Miocene to present. Many hakeas have spiny leaves with a sharp apex and/or  
106 acute, marginal teeth (Barker *et al.*, 1999). Spiny hakea leaves are more effective at deterring  
107 herbivory by kangaroos than broad leaves (Hanley *et al.*, 2007), and are moderately effective  
108 at deterring black cockatoos from reaching the woody fruits of hakeas that contain highly  
109 nutritious seeds (Groom and Lamont, 2015). Macropods appeared from 17 Ma (Prideaux and  
110 Warburton, 2010), soon after the evolution of *Hakea*. The median stem of black cockatoos  
111 (Cacatuidae, Calyptorhynchinae) is positioned at 21.5–15 Ma (White *et al.*, 2011). Needle  
112 leaves lend themselves to termination by a sharp apex, so once they appeared selection  
113 pressure from vertebrate herbivores/granivores would have promoted the evolution and  
114 stabilization of sharp-tipped leaves among vulnerable lineages.

115 Pollinator-driven speciation has been invoked to explain plant richness in some biodiversity  
116 hotspots, since pollinator shifts usually provide effective barriers to gene flow, thereby  
117 contributing to the origin of new plant lineages (van der Niet *et al.*, 2014). Hanley *et al.*  
118 (2009) concluded that insect pollination was ancestral in *Hakea* followed by repeated bouts of  
119 bird pollination. From their molecular phylogeny of 51 *Hakea* species, mainly from eastern  
120 Australia, Mast *et al.* (2012) concluded the reverse. Either interpretation is possible since it is  
121 now known that honeyeaters (Meliphagidae) originated 23.5 Ma, though they only radiated

122 strongly from 15 to 5 Ma (Joseph *et al.*, 2014). We hoped to resolve this disagreement by  
123 adding more West Australian species to our analysis. We expected bird pollination to  
124 stabilize quickly as Toon *et al.* (2014) showed for bird-pollinated legumes that this was an  
125 irreversible process.

126 Hakeas produce woody follicles that vary greatly in size (20–40450 mg) (Groom and Lamont,  
127 1997). Their seeds are highly nutritious (Groom and Lamont, 2010). By far the most  
128 destructive granivore of hakeas in southwestern Australia is Carnaby's black cockatoo  
129 (*Calyptorhynchus latirostris*, Stock *et al.* 2013). Two ways of dealing with granivores have  
130 been observed: small, camouflaged fruits that are difficult to detect visually and large,  
131 exposed fruits that resist attack mechanically (Groom and Lamont, 1997). Thus, we  
132 hypothesize that fruit size has taken two directions in *Hakea*: fruits have become either  
133 smaller (and protected or cryptic within spiny foliage, Hanley *et al.*, 2009) or larger and  
134 woodier and that these transitions are strongly unidirectional.

135 Recurrent fire is one of the key factors to high species richness in fire-prone ecosystems  
136 (Cowling *et al.*, 1996; Simon *et al.*, 2009). Whole-plant responses to fire can be placed into  
137 two regeneration syndromes: resprouters that survive fire and recover via dormant buds or  
138 meristems protected beneath the bark of trunks or underground organs (Clarke *et al.*, 2013),  
139 and nonsprouters that are killed by fire and population regeneration relies solely on seedlings.  
140 Both trait-types are well represented among hakeas (Groom and Lamont, 1996b).  
141 Nonsprouters live for a shorter time and have a higher fecundity than resprouters (Lamont &  
142 Wiens, 2003; Pausas & Verdu, 2005), and are therefore hypothesized to have higher  
143 speciation rates (Wells, 1969). Resprouters have an adaptive advantage over nonsprouters  
144 when a) fire is either frequent, rare or highly stochastic, b) conditions do not favor growth or  
145 seed production e.g. infertile soils or intense competition (fertile soils, high rainfall), and/or c)  
146 conditions do not favor seedling recruitment or adult survival (Ojeda, 1998, Lamont *et al.*,

147 2011). As the climate became more seasonal through the Miocene, the accumulation of dry  
148 matter would have been promoted and fires would have become less stochastic and more  
149 likely to occur within the lifespan of the shorter-lived nonsprouters, essential for their  
150 promotion (Enright *et al.*, 1998a) but not necessarily at the expense of resprouters (Enright *et*  
151 *al.*, 1998b). Increasing occurrence of arid periods (glacials) would have created mosaics of  
152 deep sands and exposed laterites and granites (Glassford and Semeniuk 1995) favoring  
153 nonsprouters and resprouters respectively (Lamont and Markey, 1995). Thus, existing  
154 knowledge does not enable us to predict the ancestral regeneration strategy of *Hakea*.  
155 Whatever trait was ancestral we can speculate that some of its descendants must soon have  
156 transitioned to the other trait, and transition and stabilization rates in both would have been  
157 similar throughout the Neogene-Quaternary.

158 Serotiny (on-plant seed storage) is adaptive under conditions that restrict annual seed  
159 production (poor soils, low rainfall) in fire-prone environments (for cueing seed release) with  
160 a reliable wet season (for effective seedling recruitment) (Lamont *et al.*, 1991, Cowling *et al.*,  
161 2005). While plant death from drought is sufficient to induce seed release among hakeas,  
162 postfire conditions are still required for optimal seedling recruitment (Causley *et al.*, 2016).  
163 Thus, we postulate that strong serotiny is the ancestral condition in *Hakea* and that  
164 stabilization will be the main evolutionary process for this trait in its subsequent  
165 diversification in response to intensifying fire and seasonality. Loss of serotiny will be a later  
166 development corresponding to the gradual appearance of fire-free habitats. Hakeas that  
167 migrate to summer-rainfall grasslands (savannas) that developed in the late Miocene can also  
168 be expected to become nonserotinous.

169 In this study, using a time-based phylogeny for *Hakea* we assembled (El-ahmir *et al.*, 2015),  
170 we reconstructed the ancestral traits for six attributes (with 15 trait states) and determined  
171 their evolutionary trajectories in response to the advent or increasing presence of fire,

172 seasonality, aridity, nectar-feeding birds and vertebrate/herbivores/granivores. We attempted  
173 to identify traits of the putative ancestor and the relative contribution of transition and  
174 stabilization processes to the frequency of alternative traits over geological time to account  
175 for trait representation among the extant species. Our objective was to evaluate the impact of  
176 changing environmental conditions on trait evolution and their contribution to diversification  
177 in *Hakea* to provide insights on the factors and processes explaining high species richness in  
178 this prominent Australian genus.

## 179 **MATERIALS AND METHODS**

### 180 *Phylogenetic reconstruction*

181 We built a time-based *Hakea* phylogeny (El-Ahmir *et al.*, 2015). Briefly, we included 82  
182 *Hakea* species, each with eight gene sequences extracted from NCBI (Mast *et al.*, 2012),  
183 combined with new sequences that we generated. The outgroup included *Grevillea juncifolia*,  
184 *Finschia chloroxantha* and *Buckinghamia celsissima* and their DNA sequences were obtained  
185 from NCBI. We set the calibration point for the origin of the subfamily Grevilleoideae (to  
186 which *Hakea* belongs) at 70.6 Ma based on the fossil *Lewalanipollis rectomarginis* used by  
187 Sauquet *et al.* (2009). We used BEAST v2.1.0 to estimate phylogeny and divergence times  
188 under a strict clock model (Drummond *et al.*, 2006), and further details on the methods are  
189 provided in El-Ahmir *et al.* (2015).

### 190 *Trait data*

191 We collated leaf shape and spinescence from Barker *et al.* (1999), Hanley *et al.* (2009),  
192 personal field observations and database of the State Herbarium of Western Australia  
193 (<http://www.flora.sa.gov.au>). Needle leaves were recognized as rounded in cross-section with  
194 a length:width ratio of >20:1. Heteroblastic species, with seedling leaves initially broad  
195 becoming needle by the end of the first growing season or seasonally broad to needle (Groom



196 *et al.*, 1994a), were also identified. Blunt leaves had a mucro or marginal teeth with  
197 length:width ratio <1:1 while sharp leaves were >2:1.

198 For pollinator types, Hanley *et al.* (2009) showed that stigma–nectary distance (SND) in  
199 *Hakea* is a reliable predictor of pollinator class (also adopted by Mast *et al.* 2012). All known  
200 or putative insect-pollinated species have a SND <13 mm and all known or putative bird-  
201 pollinated species have a SND >13 mm. This is supported by the shortest bill length of the  
202 principal bird pollinators in Australia (family Meliphagidae) is 12 mm (Paton and Ford, 1977)  
203 while no known insect pollinator in Western Australia can touch the nectary and pollen  
204 presenter simultaneously if the SND >12 mm. We therefore assigned species with SND <13  
205 mm to the insect-pollination class and >13 mm to the bird-pollination class. We took SND  
206 from Hanley *et al.* (2009) and Mast *et al.* (2012). Approximate SND for the remaining  
207 species were obtained from pistil lengths in Barker *et al.* (1999).

208 Fruit size, as dry fruit weight, was obtained from Groom and Lamont (1997). If not available  
209 there, the three mean fruit dimensions were obtained from Barker *et al.* (1999), converted to  
210 volume and multiplied by mean fruit density in Groom and Lamont (1997). They were  
211 divided into four size classes: <1, 1–5, >5 g, such that the 1–5 g class accounted for about  
212 half of species. Postfire response/regeneration strategy was collated from Groom and Lamont  
213 (1996b), Barker *et al.* (1999) and Young (2006). Each species was assigned as either a  
214 nonsprouter or resprouter (with two species recognized to have both fire response forms in  
215 different populations). Level of serotiny was obtained from Groom and Lamont (1997) and  
216 images on the web (especially <http://www.flora.sa.gov.au>).

#### 217 *Trait reconstruction through the phylogeny*

218 We used MultiState in BayesTraits (Pagel and Meade 2006) to determine the most likely  
219 ancestral traits for the *Hakea* phylogeny. First, we tested which of the possible models

220 (simple or complex, associated with uniform rates of 0~30) should be used via the log Bayes  
221 factor (log BF) recommended by Pagel and Meade (2006). We excluded morphological data  
222 for the outgroup in order to avoid potential biases in trait assignment because they do not  
223 adequately represent the associated clades. We applied the best-fit model parameters to our  
224 MC tree in a Bayesian framework using MCMC sampling to search for optimal parameter  
225 estimates. The MCMC parameter searches consisted of 1,000,000 iterations with 25,000  
226 discarded as burn-in. We used maximum likelihood parameter estimates as starting values in  
227 the MCMC analyses. We also used the continuous random walk (Model A) associated with  
228 the MCMC method to determine whether pairwise traits evolved in a correlated manner, and  
229 BayesFactor was used to determine the significance of correlation between any two traits  
230 (Pagel and Meade, 2006). Trait reconstruction of fruit size was carried in Mesquite using a  
231 parsimonious procedure (Madison *et al.*, 2007).

### 232 *Speciation and trait proliferation rates*

233 Net species diversification rate was calculated as  $(N_{i+t} - N_i)/(N_i \cdot t)$ , where  $N$  is the number of  
234 lineages at the start,  $i$ , and end,  $i + t$ , of the time interval,  $t$  (He *et al.* 2011) for the three  
235 geological periods/epochs in which *Hakea* has been recorded as well as overall. The  
236 geological boundaries were set according to the International Commission on Stratigraphy  
237 ([www.stratigraphy.org](http://www.stratigraphy.org)), while the start time in the Miocene was set at the time that *Hakea*  
238 first appeared. Following trait assignment to each node of the phylogeny, trait stabilization  
239 and transition rates (see Introduction) were determined for the three periods/epochs and  
240 overall by counting their number in each time interval. Where the ancestor was ambiguous  
241 this event was omitted from the counts as the process was unclear. They were then converted  
242 to the fraction that each process contributed to total proliferation within the trait and between  
243 all traits of that character. The number of reversals was also noted: i.e. a trait reverting to its  
244 immediate preceding trait. Individual speciation promotional rates (SPR) for the three

245 geological periods were determined from the percentage of events retaining the ancestral trait,  
246 Y, where  $SPR = 100/Y$  (see Introduction). Generally,  $SPR_n = \prod_{i=1}^n 100/Y_i$  where Ys for the  $n$   
247 attributes assessed are multiplied to give their total promotional effect on species  
248 diversification.  $SPR_n$  was converted to its fractional contribution to species diversification for  
249 the  $n$  attributes assessed:  $(SPR_n - 1) / SPR_n$ .

## 250 **RESULTS**

### 251 *Hakea time-based phylogeny*

252 The Bayes MCMC analysis indicated that the *Hakea* stem arose 18.0 Ma [with the 95%  
253 highest density probability (HDP) at 15.8–20.2 Ma] and split into two clades (defined as  
254 clades A and B in Mast *et al.* 2012) 14.1 Ma (95% HPD, 12.5–15.8 Ma). The phylogeny was  
255 strongly supported by the branch posterior probability where 48 out of 81 branches were  $\geq$   
256 0.70. The overall topology of *Hakea* phylogeny was consistent with that in Mast *et al.* (2012).  
257 Net species diversification rate in the Miocene greatly exceeded that in the Pliocene (9.6 $\times$ )  
258 and Quaternary (13.5 $\times$ ) and the overall rate was dictated by the Miocene rate as it was the  
259 longest period (Table S1).

### 260 *Evolutionary trajectories for two leaf attributes*

261 Trait reconstruction showed that the most recent ancestor (MRA) had broad leaves ( $P = 0.61$ )  
262 that were blunt-tipped with smooth margins ( $P = 0.88$ ) (Fig. 2). The phylogeny split into  
263 needle (A) ( $P = 0.78$ ) and broad (B) ( $P = 1.00$ ) clades by 14.1 Ma. Heteroblasty arose 6.9 Ma.  
264 Both clades remained blunt-tipped ( $P = 0.69, 1.00$ ) but sharp tips emerged in one branch of  
265 the A clade 12.7 Ma. While the transition rate for needle/heteroblastic leaves exceeded that of  
266 broad leaves in the Miocene, proliferation of broad leaves accounted for 60% of the  
267 diversification events (Table S1A). Broad leaf proliferation continued (mainly through  
268 stabilization) at the expense of needle but not of heteroblastic leaves through the Pliocene and

269 Quaternary. Overall, 65% of total proliferations were of broad leaves (mainly stabilization)  
270 with 33 reversals to broad leaves, 30% to needle and 5% to heteroblastic (mainly recent  
271 transitions), with the overall transition rate of non-broad leaves 2.6 times broad leaves. The  
272 evolution of non-broad leaves increased the overall speciation rate by 54%, greatest in the  
273 Miocene. Spiny leaves proliferated at a similar rate as non-spiny leaves in the Miocene but  
274 the rate for spiny leaves declined slightly through the Pliocene and Quaternary due to  
275 reducing stabilization but increased slightly among non-spiny leaves due to increasing  
276 stabilization (Table S1B). Reversals were negligible. The transition rate for spiny leaves was  
277 twice that for non-spiny leaves, with their advent and proliferation increasing the  
278 diversification rate by 73%.

#### 279 *Evolutionary trajectories for two reproductive attributes*

280 The MRA of *Hakea* showed a high posterior probability ( $P = 0.75$ ) of being insect-pollinated.  
281 The basal split of the genus was accompanied by a shift to bird pollination 14.1 Ma in clade B  
282 ( $P = 0.82$ ) but retention of insect pollination in clade A ( $P = 0.98$ ) (Fig. 3). A reversal  
283 occurred in clade B 12.6 Ma while pollination transitioned to birds 12.1 Ma in clade A that  
284 remained predominantly insect-pollinated. Overall, 78 reversals occurred (Table S1C). The  
285 switch to bird pollination was restricted to the Miocene with transitions accounting for 32%  
286 of bird proliferation events, and increasing stabilization through the Pliocene/Quaternary.  
287 Bird to insect transitions occurred in the Pliocene but not in the Quaternary. Overall transition  
288 rates for insect and bird pollination were similar, with bird pollination accounting for 30% of  
289 events and promoting speciation by 41%. The MRA had a high probability (by parsimony) of  
290 producing medium-sized fruits (1.0–5.0 g). Smaller (<1.0 g) and larger (>5.0 g) fruits first  
291 arose 12.1 Ma in clade A and smaller fruits appeared 6.5 Ma in clade B (Table S1D). In the  
292 Miocene, 19% of events involved transitions to other than medium-sized fruits but  
293 proliferation of medium-sized fruits predominated. Proliferation of small fruits (46% of

294 events) dominated in the Pliocene, through both transitions and stabilization, and proliferation  
295 of non-medium-sized fruits contributed 150% to the stimulation of diversification events. In  
296 the Quaternary and overall, proliferation of medium and non-medium fruits contributed  
297 equally to all diversification events. Only medium fruits were sometimes the outcomes of  
298 reversals; all other transitions were unidirectional with medium→small accounting for 30  
299 events, medium→medium-large/large for 11 events, and medium→medium-large→large for 8  
300 events. Overall, 24% of all events involved transitions to non-medium fruits and their  
301 proliferation accounted for an 88% increase in the speciation rate.

### 302 *Evolutionary trajectories for two fire-adapted attributes*

303 Postfire regeneration of the MRA was via resprouting though the posterior probability was  
304 not strong ( $P = 0.62$ ). The ancestor of clade A was a resprouter ( $P = 0.73$ ), while clade B was  
305 a nonsprouter ( $P = 0.86$ ) (Fig. 4). By 12.7 Ma, nonsprouters also evolved in clade A. By the  
306 end of the Miocene, diversification events were spread almost uniformly between resprouters  
307 and nonsprouters (Table S1E). Transitioning to nonsprouters remained strong in the Pliocene  
308 but ceased among resprouters. Transitioning ceased in the Quaternary with nonsprouting  
309 promoting 140% more speciation through stabilization in that period. Overall, proliferation  
310 among resprouters and nonsprouters was similar with the advent of nonsprouters doubling the  
311 speciation rate due to similar high rates of stabilization, though transitions to nonsprouting  
312 approached twice that for resprouting. Reversals were common among resprouters but only  
313 20% of reversals involved nonsprouters. Serotiny was the MRA with  $P = 1.00$  and both major  
314 clades remained serotinous ( $P = 1.00$ ). There was an isolated occurrence of weak/nil serotiny  
315 12.1 Ma and five more subsequent origins in clade A but non-serotiny never arose in clade B.  
316 Stabilization among moderately/strongly serotinous lineages dominated trait proliferation  
317 throughout hakea's history with limited transition to weak/non-serotiny in the Miocene  
318 followed by stabilization in the Pliocene and absence of proliferation in the Quaternary.

319 Overall, stabilization of serotiny was the main process with proliferation of non-serotiny  
320 accounting for 7% of events and it increased speciation by 8%. All transitions were  
321 unidirectional.

### 322 *Promotion of species diversification*

323 The overall speciation promotional rate ( $SPR_6$ ) induced by the advent of novel traits was  
324 given by  $1.54 \times 1.73 \times 1.41 \times 1.85 \times 2.02 \times 1.08 = 15.16$ . Thus, 93.4% of diversification  
325 events (ignoring reversals) can be attributed to the presence of at least one non-ancestral trait.  
326 Three species possessed the six ancestral traits (*H. candolleana*, *H. ceratophylla*, *H.*  
327 *eriantha*), all in the same subclade of 14 species, but they included two reversals  
328 (nonsprouter→resprouter, medium-large→medium fruits). Thus, 96.3% of extant species lack  
329 at least one ancestral trait. One species (*H. divaricata*) had five of six traits in the advanced  
330 condition.

### 331 *Correlated evolution between traits*

332 Correlation analysis using the BayesFactor (BF) showed no relationship between any pairs of  
333 attributes ( $BF < 1.0$ ) except leaf shape and spinescence, with needle leaves more likely to be  
334 spiny ( $BF = 5.3$ ).

## 335 **DISCUSSION**

336 Trait reconstruction of the ancestral *Hakea* phenotype shows it to have been broad-leaved,  
337 non-spinescent and insect-pollinated, with medium-sized, serotinous fruits and resprouting  
338 after fire. Resprouting and serotiny confirm that the associated vegetation was fireprone and  
339 experienced a reliable postfire wet season by 18 Ma (Lamont *et al.*, 2013). It is clear that  
340 *Hakea* changed radically at the level of fire-related adaptations, including woodiness of their  
341 fruits, when migrating from the non-fireprone environment of its ancestors (nonsprouting and

342 nonserotinous), whereas leaf form (broad, non-spinescent) and reproductive biology [insect-  
343 pollinated, medium-sized (1–5 g) fruits] were initially conserved. Nevertheless, within 4 My,  
344 two (sub)clades had evolved with quite different syndromes of traits: one (A) that retained  
345 resprouting but possessed needle leaves many of which developed sharp apices, was bird-  
346 pollinated and where the largest woody fruits (>10 g) were produced, and the other (B) that  
347 became nonsprouting but all other attributes were dominated by their ancestral traits. The  
348 final outcome was almost equal representation of broad and needle leaves and spiny and blunt  
349 leaves, significant presence of bird-pollination, almost equal representation of small (<1 g)  
350 and large (>5 g) fruits, equal representation of resprouting and nonsprouting, and limited  
351 presence (10%) of weak/non-serotiny. Only three of 82 extant species retain all six ancestral  
352 traits and even two of these traits were the outcome of reversals. At the genus level, of 15  
353 possible pairs of correlated evolution between attributes, only needle and sharp-pointed  
354 leaves were associated through time (attributable to their morphological links).

355 The species diversification rate of *Hakea* was highest by far in the Miocene than in the more  
356 recent epochs. The Miocene was a period of great climatic upheavals and the speciation rates  
357 among banksias in Australia (He *et al.*, 2011) and proteas in South Africa (Lamont *et al.*,  
358 2013) (both genera also in Proteaceae) were also an order of magnitude higher than. The same  
359 pattern applies to proliferation of traits, with all alternative traits of the six examined highest  
360 in the Miocene (obtained by multiplying the percentage contribution to species diversification  
361 of each trait by the diversification rate on a time basis).

### 362 *Transition versus stabilization processes*

363 While trait initiation (transition) is a vital step in speciation its incorporation into the clade  
364 (stabilization) is just as important. That proliferation of a trait through the phylogeny is rarely  
365 a function of the transition rate is strongly supported here. Taking leaf shape as an example,  
366 the transition from broad to needle leaves overall occurred at >2.6 times the rate as the

367 reverse transition, yet stabilization of broad leaves occurred at 2.3 times the rate as needle  
368 leaves. The net result was the proliferation of broad leaves at 1.85 times the rate of needle  
369 leaves because the ratio of stabilization to transition events among broad leaves was five  
370 times the rate for needle leaves. In theory, only one initiation step is required for  
371 incorporation of a new trait into the clade provided it stabilizes quickly and is not subject to  
372 reversals. Thus, the ratio of stabilisation to transition events is a function of the strength of  
373 directional selection. The invasion of the savanna grasslands by *Protea* is a rare example of  
374 unidirectional selection associated with a single transition followed by almost universal  
375 stabilization (Lamont *et al.*, 2013). In practice, the same trait arises numerous times through  
376 the phylogeny while reversals depend on the trait. For *Hakea* leaf shape, 77% of the 43-  
377 recorded reversals were for the recovery of broad from needle leaves rendering transitions to  
378 needle less effective and reflecting unstable selective forces. The relative contribution of  
379 transition and stabilization events to proliferation depends on both the trait and the time  
380 period under consideration.

### 381 *Evolutionary trajectories for leaves*

382 By the time *Hakea* separated from its non-fireprone ancestors 18 Ma, Australia (as much of  
383 the world) was experiencing declining levels of rainfall, temperatures and metabolically  
384 active atmospheric gases, and increasing seasonality. In addition, the opening up of the  
385 vegetation would have exposed them to high light intensity and diurnal temperatures (Jordan  
386 *et al.*, 2005) compared with closed forests. If needle leaves increase fitness to such  
387 constraints, and currently they account for 45% of species so this genus has a strong  
388 propensity to produce them, they should have evolved early in its history and proliferated  
389 through stabilization. Indeed, within 4 My, a needle-leaved clade (A) had arisen with strong  
390 stabilization leading to 56% of its extant species being needle-leaved. Evolution of needle  
391 leaves was greatly delayed in clade B and was mainly expressed through the appearance of



392 heteroblastic species over the last 5 My (all from broad-leaved ancestors). The latter appeared  
393 so recently that there have been no opportunities for reversals unlike needle leaves where  
394 reversals to broad have been frequent. These reversals confirm the lability of leaf form among  
395 isobilateral leaves as demonstrated ontogenetically by *H. trifurcata* whose juvenile leaves are  
396 needle, a few becoming broad at the start of the growing season in adult plants and needle  
397 again as the dry summer approaches (Groom *et al.*, 1994a).

398 The dominance of broad leaves and reversals to them require some explanation. Clearly, leaf  
399 form is not the only way of dealing with drought, such as deep root systems (Groom and  
400 Lamont, 2015), while broad leaves among hakeas are still highly sclerophyllous (Lamont *et*  
401 *al.*, 2015) with thick cuticles, sunken stomata and a tannin-filled hypodermis (Jordan *et al.*,  
402 2005). They are often narrow or strap-shaped rather than truly broad, such as *H.*  
403 *grammatophylla* in the 'deadheart' of Australia, and all are vertically oriented. In addition,  
404 broader-leaved species have retreated to the moister parts of the landscape or subregions  
405 (Groom and Lamont, 1996a). In fact, the frequent reversals in both directions are consistent  
406 with climatic oscillations that became characteristic of the Pliocene/Quaternary and their  
407 evolutionary tracking.

408 While broad leaves may be spinescent, such as *H. cristata*, needle leaves that are already rigid  
409 and with a sclerified apex can readily be transformed into strongly piercing structures through  
410 elongation and thinning of the mucro. This morphogenetic link explains the unique  
411 evolutionary correlation through time of needle and spinescent leaves. Thus, following a  
412 small delay, one branch of the A clade became spinescent at 12.7 My. The broad-leaved B  
413 clade remained essentially non-spinescent. If spinescence is effective against herbivores  
414 (macropods) and florivores/granivores (emus, cockatoos) (Hanley *et al.*, 2007) the delay in its  
415 appearance cannot be attributed to their absence as all were present by this time but they  
416 speciated gradually and their selective effects would have intensified over time. It is of

417 interest that transitioning to spiny leaves was most marked in the Quaternary, a time when  
418 modern cockatoos evolved in SW Australia, though their ancestors were present from the  
419 early Miocene (Joseph *et al.*, 2014). Reversals were negligible indicating strong directional  
420 selection. Why more events did not yield spiny leaves is partly attributable to morphological  
421 constraints, the fact that all *Hakea* leaves are highly unpalatable and not grass-like (Rafferty  
422 *et al.*, 2010), and ability of vertebrates to learn to overcome physical deterrents (Hanley *et al.*,  
423 2007).

#### 424 *Evolutionary trajectories for flowers and fruits*

425 Bird-pollinated flowers evolved from insect-pollinated flowers with the split of the genus  
426 14.1 Ma. This resolves the disagreements over which was the ancestral condition (Hanley *et*  
427 *al.*, 2009; Mast *et al.*, 2012) caused by misidentifying the basal lineages or not including  
428 sufficient (representative) insect-pollinated lineages from SW Australia where the clade most  
429 probably arose (Lamont *et al.*, 2016). Honeyeaters (Meliphagidae) were already present in  
430 Australia at the time *Hakea* originated, but these birds only diversified strongly in the mid to  
431 late-Miocene, especially among such major pollinators as *Phylidonyris*, *Anthochaera*,  
432 *Lichmera* and *Lichenostomus* (Joseph *et al.*, 2014). In fact, apart from *H. cucullata* in the  
433 Quaternary, the only time flowers increased their size to accommodate bird pollinators was in  
434 the Miocene. For the Pliocene/Quaternary it was stabilization processes only. The greater  
435 levels of stabilization among insect-pollinated lineages throughout their history explains their  
436 current greater abundance and suggests that they have been a greater selective force,  
437 possibility associated with their greater reliability rather than morphological diversity that  
438 would have favored greater transition rates.

439 Of note are the transitions from bird to insect pollination in the Miocene/Pliocene and the  
440 large number of reversals (78), 60% of which were insect→bird→insect. This is significant on  
441 two counts: trait reversibility and fluctuating selection. Bird-pollination is regarded as an

442 evolutionary 'dead-end' because specialization of floral structures for birds may be  
443 irreversible (Toon *et al.*, 2014) so shifts from bird to insect pollination are rare (van der Niet  
444 & Johnson 2012). This hypothesis derives from hummingbird pollination systems that require  
445 specialised floral structures (Tripp and Manos, 2008). However, specialised floral structures  
446 are not essential for honeyeaters because they are generalist pollinators and not obligate  
447 nectarivores. This lack of specialization in the honeyeater pollination system implies minimal  
448 floral structure specialization – simple elongation of the pistil is sufficient (Hanley *et al.*,  
449 2009). As a result, reversal shifts from bird to insect are possible in situations when bird  
450 pollinators become scarce. The evolutionary tracking of the great climatic fluctuations, with  
451 their profound effects on the abundance of both birds and insects, that occurred from the mid-  
452 Miocene can explain the remarkable number of reversals in both directions. Such great  
453 flexibility in pollinator shifts may provide insights in explaining the mechanisms that  
454 promoted explosive speciation in *Hakea* but even more so in its younger sister, *Grevillea* with  
455 150 of 362 species being bird-pollinated (Ford *et al.*, 1979).

456 Morphological variation in fruit structure among hakeas is exceptional and transition rates  
457 away from the ancestral medium-sized ancestor equalled their stabilization rates through each  
458 of the three epochs. Two groups of granivores, insects and parrots, were already present when  
459 *Hakea* emerged. The transition to small (camouflaged) fruits, which appear better protected  
460 against insects than cockatoos (B Lamont, unpublished), was strongest in the Miocene and  
461 continued throughout the period, by far the highest among all traits we assessed. Large woody  
462 fruits, particularly effective against cockatoos (Groom and Lamont, 2015), followed a similar  
463 path. There were no reversals among small and large fruits indicating strong unidirectional  
464 selection for these extremes.

#### 465 *Evolutionary trajectories for fire-related traits*

466 The twin ancestral traits of resprouting (adaptive in the presence of severe, periodic

467 disturbances where recruitment opportunities are limited) and serotiny (ensuring seeds are  
468 released when those limited recruitment opportunities are optimal) demonstrate that *Hakea*  
469 arose in a fireprone environment. We can also surmise that fires were of overall moderate  
470 frequency (>5–45 y intervals) but highly stochastic. If fires were at the high frequencies  
471 associated with savanna grasslands (<5 y intervals) the plants would have stayed  
472 nonserotinous (Lamont *et al.* 2013). If fire intervals exceeded their longevity then, upon death  
473 in the absence of fire, serotinous seeds would have been released onto a hostile seedbed and  
474 rarely yielded recruits (Causley *et al.*, 2016). However, within 4 My, a nonsprouting clade (B)  
475 had arisen. The outcome was strong transitioning to both fire-response types in the Miocene  
476 and all epochs were dominated by stabilizations against a background of almost universal  
477 proliferation by serotinous descendents. Of note are both the continuing transitions to  
478 nonsprouting in the Pliocene and its steady increase in stabilization rate throughout *Hakea's*  
479 history. It is likely that the trend of increasing aridity and seasonality and declining  
480 atmospheric oxygen and carbon dioxide (He *et al.*, 2012) led to less frequent, but more  
481 reliable, fires and promotion of nonsprouting (Lamont *et al.*, 2011, 2013). Today, resprouters  
482 are better represented in the more fireprone, strongly seasonal northern sandplains of SW  
483 Australia and nonsprouters in the less fireprone parts, especially on deeper soils where  
484 recruitment and adult survival are more likely (Lamont and Markey, 1995; Groom and  
485 Lamont, 1996a).

486 Transitions to non/weak serotiny were rare but five of the six independent origins were in the  
487 late Miocene and proliferation was restricted to the Pliocene. Explanations vary but include  
488 migrations to frequently-burnt savanna grasslands (*H. divaricata* lineage) or rarely-burnt  
489 aridlands (*H. recurva*), exposure of novel firefree rock outcrops to which some species  
490 adapted (*H. clavata*), and presence in forests with reliable winter rains ensuring recruitment  
491 interfire (*H. trifurcata* lineage) (Hanley and Lamont, 2001). This pattern has limited parallels  
492 with *Protea* in South Africa where one transition to nonserotiny in grasslands was followed

493 by increasingly extensive stabilization there that failed to occur in Australian grasslands.  
494 However, in both super-regions stabilization of the ancestral condition, serotiny, was by far  
495 the dominant process and reversals were negligible. By contrast, the well known lability in  
496 whole-plant fire responses (He *et al.*, 2011) was expressed in both *Protea* and *Hakea*,  
497 although 80% of reversals were to resprouting in *Hakea* in the Miocene, perhaps reflecting  
498 periods of increased or more stochastic fire frequencies as the clade moved to other parts of  
499 Australia. The historic levels of fire frequency coupled with the severity of seasonal droughts  
500 serve well to interpret the relative abundances and distributions of resprouters and  
501 nonsprouters among hakeas, ericas and proteas (Ojeda, 1998; Lamont *et al.*, 2013).

#### 502 *Promotion of speciation by non-ancestral traits*

503 There are two ways of considering the role of traits in speciation. One is to compare how they  
504 have *contributed to* all diversification events and the other is to estimate to what extent non-  
505 ancestral traits have *promoted* these diversification events. The latter is hypothesis-driven and  
506 based on the premise that without trait innovation and subsequent stabilization these  
507 diversification events would not have occurred. Thus, it relies on being able to identify both  
508 the ancestral state and its pathway through the phylogeny in order to ascertain trait reversals.  
509 This means that any extinction events cannot be incorporated into the analysis, but we argued  
510 earlier (see Introduction) that most new traits are adjunct to (as they are spatially displaced),  
511 rather than replace, ancestral traits anyway. Nevertheless, entire but unknown lineages  
512 characterized by certain historically maladapted traits may be missing (or only represented by  
513 long branches in the chronogram) if there have been radical environmental shifts that no  
514 amount of adjustment, in the absence of fossil evidence, can correct for. However, there is  
515 little evidence of extinctions as a significant evolutionary process in the SW Australian flora  
516 over the last 10 My (Hopper, 2009).

517 For the six *Hakea* attributes examined, proliferation of the non-ancestral trait promoted  
518 speciation by 1.08 (weakly serotinous) to 2.02 (fire-killed) times. Overall, this increased  
519 speciation by 15.2 times, equivalent to 93.4% of diversification events. Two of the ancestral  
520 traits possessed by the three species of identical phenotype to the putative original phenotype  
521 were the result of reversals so that even these were the outcome of trait diversification when  
522 the ancestral condition would have been temporarily lost. Reversals were particularly  
523 prevalent among pollination types and fire-response types but absent altogether from the  
524 serotiny types. They represent the net effect of a) the constancy of directional selection, b) the  
525 lability of opposing traits, and c) time available for further transitions to occur. Certainly, the  
526 high level of lability among fire-response types is consistent with previous studies on *Protea*  
527 (Lamont et al. 2013), *Banksia* (He *et al.*, 2011) and Restionaceae (Litsios *et al.*, 2014).

528 Clearly, *Hakea*'s remarkable genetic/morphological malleability in the face of these strong  
529 selective agents has resulted in an exceptionally diverse clade and its distribution throughout  
530 Australia. We might now wonder to what extent the cumulative contributions by transitions  
531 and stabilizations to trait proliferation among critical plant attributes, in response to an array  
532 of environmental constraints introduced in the Miocene, have led to the explosive radiation of  
533 such speciose genera as *Grevillea*, *Acacia*, *Melaleuca* and *Eucalyptus*, that currently  
534 dominate Australia's sclerophyll flora, and the floras of many other parts of the world subject  
535 to similar selective forces.

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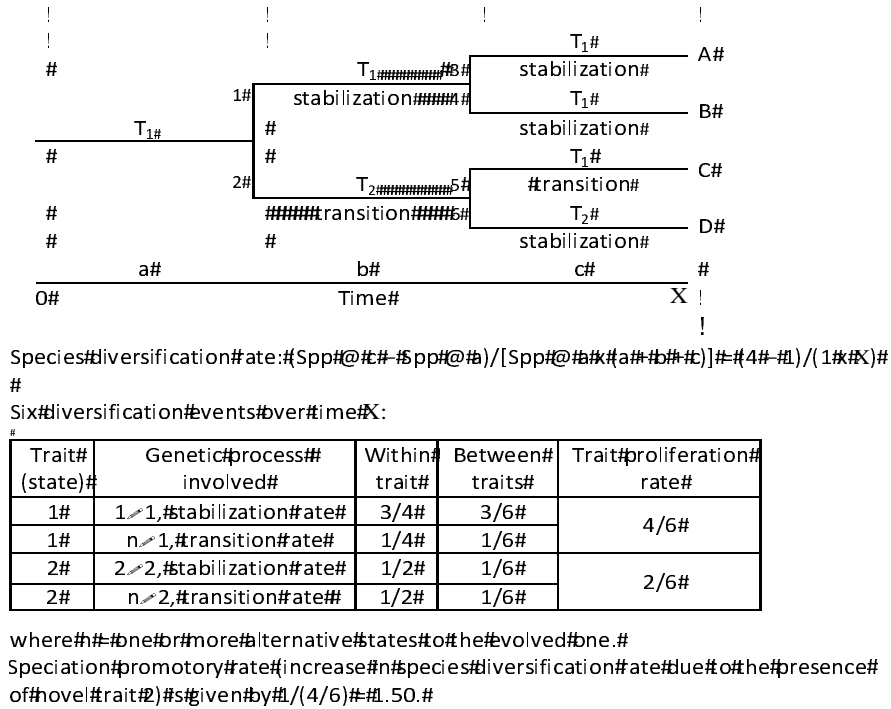
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672 **Figures**

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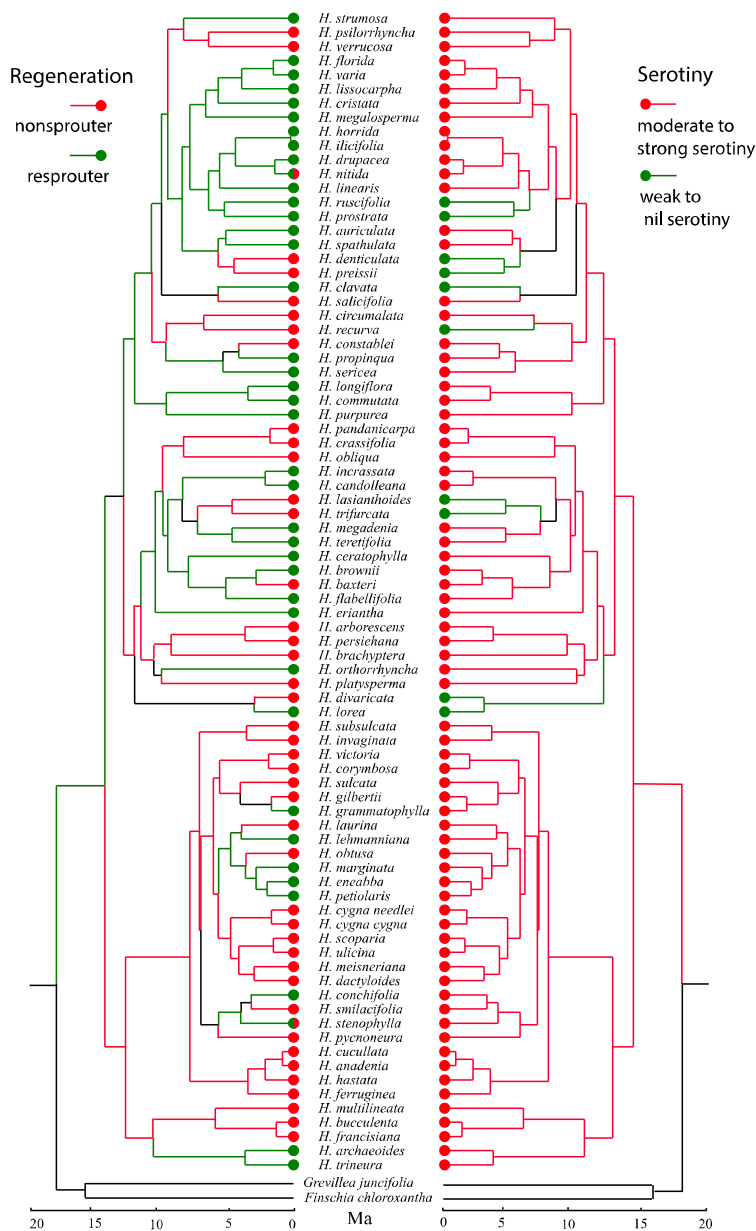
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675 Fig. 1. Hypothetical phylogeny showing the evolution of four species from six diversification  
 676 events with proliferation of two alternative traits, 1 (ancestral) and 2 (novel), of a given  
 677 character over time, due to both stabilization and transition processes. Diversification rate is  
 678 relative to the starting number of species/lineages and the time interval, while proliferation,  
 679 stabilization and transition rates are relative to the maximum number of events in which that  
 680 trait could occur. Note that the two transitions that yield Sp C is an example of a reversal (to  
 681 T<sub>1</sub>).

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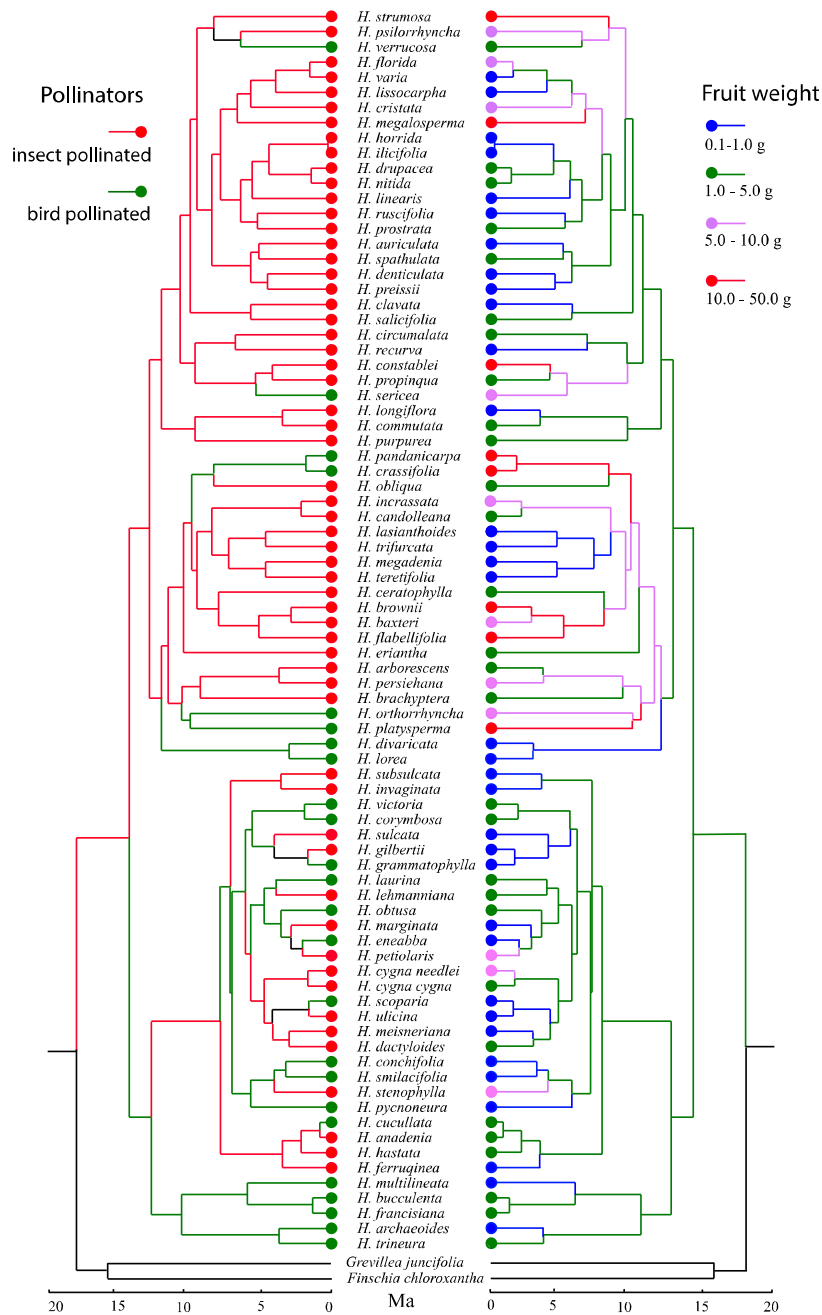


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686 Figure 2. Reconstruction of leaf morphology traits through time in the genus *Hakea*. Left: leaf  
 687 shape, broad, needle or heteroblastic (broad followed by needle). Right: leaf spinescence,  
 688 blunt/nil or sharp apices or teeth.

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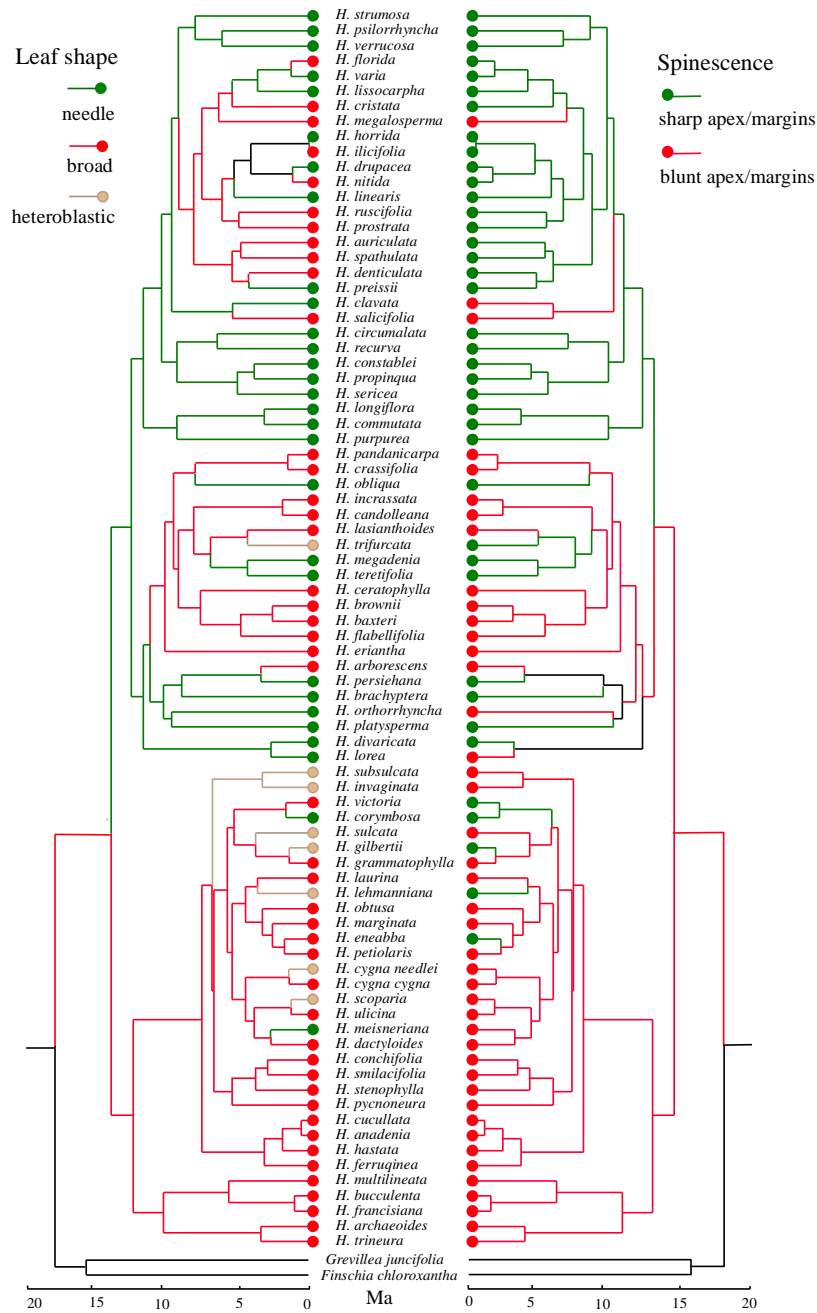


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692 Figure 3. Reconstruction of reproductive biology traits through time in the genus *Hakea*. Left:  
 693 insect or bird pollinated. Right: four classes of fruit size by weight.

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697 Figure 4. Reconstruction of fire-adapted traits through time in the genus *Hakea*. Left:  
 698 nonsprouter (seedlings only) or resprouter. Right: strongly or weakly/nil serotinous.

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Supplementary materials

Table S1. Paired trait evolution in *Hakea* apportioned among stabilization (trait retained during diversification event) and transition (trait attained during diversification event) processes in each Epoch based on the molecular chronogram reported here. All node-to-node steps in the phylogeny were treated as diversification events. Where the ancestor was ambiguous this event was omitted from the counts as the process was unclear. Reversals refer to transitions back to the previous trait.

| Epoch/period:                      |                 | Miocene (18—5.3 Ma) |                    | Pliocene (5.3—2.6 Ma) |                    | Quaternary (2.6—0 Ma) |                    | Overall (18—0 Ma) |                    | Number of reversals |
|------------------------------------|-----------------|---------------------|--------------------|-----------------------|--------------------|-----------------------|--------------------|-------------------|--------------------|---------------------|
| Species diversification rate:      |                 | 3.23                |                    | 0.23                  |                    | 0.07                  |                    | 4.48              |                    |                     |
| Trait that evolved                 | Genetic process | Within trait (%)    | Between traits (%) | Within trait (%)      | Between traits (%) | Within trait (%)      | Between traits (%) | Within trait (%)  | Between traits (%) |                     |
| <i>A. Leaf shape</i>               |                 |                     |                    |                       |                    |                       |                    |                   |                    |                     |
| Broad leaves (ancestral)           | Stabilization   | 93.9                | 56.8               | 96.9                  | 64.6               | 94.1                  | 72.7               | 94.9              | 61.6               | 33                  |
|                                    | Transition      | 6.1                 | 3.7                | 3.1                   | 2.1                | 5.9                   | 4.6                | 5.1               | 3.3                |                     |
| Needle leaves                      | Stabilization   | 87.1                | 33.3               | 83.3                  | 20.8               | 50.0                  | 4.6                | 84.4              | 25.2               | 10                  |
|                                    | Transition      | 12.9                | 4.9                | 16.7                  | 4.2                | 50.0                  | 4.6                | 15.6              | 4.6                |                     |
| Heteroblasty                       | Stabilization   | 0.0                 | 0.0                | 50.0                  | 4.2                | 0.0                   | 0.0                | 25.0              | 1.3                | 0                   |
|                                    | Transition      | 100.0               | 1.2                | 50.0                  | 4.2                | 100.0                 | 13.6               | 75.0              | 4.0                |                     |
|                                    |                 | 100.0               |                    | 100.0                 |                    | 100.0                 |                    | 100.0             |                    |                     |
| Fisher Test ( <i>P</i> , 2-tailed) |                 | 0.0367              |                    | 0.0120                |                    | 0.0013                |                    | <0.0001           |                    |                     |
| Speciation promotion rate          |                 | 1.65                |                    | 1.50                  |                    | 1.29                  |                    | 1.54              |                    |                     |

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