Origin of the endemic fern genus *Diellia* coincides with the renewal of Hawaiian terrestrial life in the Miocene

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The enigmatic fern genus *Diellia*, endemic to the Hawaiian archipelago, consists of five extant and one recently extinct species. *Diellia* is morphologically highly variable, and a unique combination of characters has led to several contrasting hypotheses regarding the relationship of *Diellia* to other ferns. A phylogenetic analysis of four chloroplast loci places *Diellia* within 'black-stemmed' rock spleenworts of the species-rich genus *Asplenium*, as previously suggested by W. H. Wagner. Using an external calibration point, we estimate the divergence of the *Diellia* lineage from its nearest relatives to have occurred at ca. 24.3 Myr ago matching an independent estimate for the renewal of Hawaiian terrestrial life (ca. 23 Myr ago). We therefore suggest that the ancestor of the *Diellia* lineage may have been among the first successful colonists of the newly emerging islands in the archipelago. Disparity between morphological and nucleotide sequence variation within *Diellia* is consistent with a recent rapid radiation. Our estimated time of the *Diellia* radiation (ca. 2 Myr ago) is younger than the oldest island of Kaua'i (ca. 5.1 Myr ago) but older than the younger major islands of Maui (ca. 1.3 Myr ago), Lana'i (ca. 1.3 Myr ago) and Hawaii (ca. 0.43 Myr ago).

Keywords: *Asplenium*; *Diellia*; island radiation; palaeo-endemic

1. INTRODUCTION

The vascular flora of the Hawaiian Islands is renowned for its unique assemblage of endemic species and lineages (Funk & Wagner 1995; Baldwin *et al.* 1998; Baldwin & Sanderson 1998; Givnish 1998; Wright *et al.* 2001; Lindqvist & Albert 2002; Palmer 2003). Island clades exhibiting greater morphological variation than their mainland relatives are well known (Funk & Wagner 1995; Baldwin *et al.* 1998; Givnish 1998). Extreme morphological divergence has often obscured the origin of Hawaiian endemics, which is important to our understanding of the origin and evolution of the Hawaiian flora. Although recent research has focused on Hawaiian angiosperms, several major endemic or nearly endemic lineages of Hawaiian ferns exist, including *Adenophorus* Gaudich. (Grammitidaceae), *Cibotium* Kaulf. (Dicksoniaceae), *Sadleria* Kaulf. (Blechnaceae) and *Diellia* Brack. (Aspleniaceae). Only *Adenophorus* have been studied using phylogenetic approaches so far (Ranker *et al.* 2003). Out of these Hawaiian fern radiations, *Diellia* Brack., with its remarkable morphological variation, is probably the most enigmatic and widely debated. This lineage of five extant species occurs in small isolated populations in montane habitats above 500 m on major

islands of Hawaii, although several species are restricted to a single island (Agurauja & Wood 2002, 2003; Palmer 2003).

The origin of the Hawaiian endemic genus *Diellia* has puzzled scholars for some time. Based on *Diellia*'s clathrate scales, Copeland (1929) rejected relationships to lindsaeoid ferns and suggested instead close relationships to the asplenioid fern genus *Loxoscaphe*. Comparative analyses of morphological features common to species of *Diellia* led W. H. Wagner to propose instead that *Diellia* was most closely related to the 'black-stemmed' group of rock spleenworts, typified by *Asplenium trichomanes* L. (Wagner 1952, 1953a,b). He identified *D. leucostegioides* (Baker) W. H. Wagner (now apparently extinct) as the 'prototype' of *Diellia* because it resembled the widespread palaeotropical species *A. normale* D. Don in most of its morphological features (Wagner 1953a).

Whereas the 'black-stemmed' rock spleenwort clade is (more or less) morphologically uniform, species of *Diellia* are remarkably variable in leaf dissection, venation and indument (Wagner 1952; Palmer 2003). Although the variation exhibited by *Diellia* is absent from the 'black-stemmed' rock spleenworts, it does occur in other lineages of asplenioid ferns: the 2–3-pinnate leaves of *D. mannii* (D. C. Eaton) W. J. Rob. resemble leaves found in the South American *Asplenium cristatum* Lam., and the regularly anastomosing veins of *D. falcata* Brack. and *D. pallida*

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W. H. Wagner resemble venation patterns found in the *Camptosorus* L. and *Ceterach* L. clades of *Asplenium* L.

We used molecular data to: (i) identify the nearest living relatives of *Diellia* and clarify the origin of this fascinating group, (ii) provide age divergence estimates for the *Diellia* lineage, and (iii) evaluate the (adaptive) radiation of *Diellia* in the Hawaiian islands.

2. MATERIAL AND METHODS

(a) Datasets

We sampled extensively across asplenoid ferns with particular emphasis on species of the 'black-stemmed' rock spleenwort clade. We included one individual from each extant *Diellia* species as well as the putative hybrid *D. × lauii* (*D. falcata* × *D. unisora*). Three *Hymenasplenium* Hayata species served collectively as the outgroup, as recent phylogenetic studies provided convincing evidence for two major evolutionary lineages in asplenoid ferns corresponding to the genera *Asplenium* and *Hymenasplenium* (Murakami *et al.* 1999; Gastony & Johnson 2001; Schneider *et al.* 2004a). Electronic Appendix A includes voucher information, along with GenBank accession numbers, for all taxa sampled in this study.

(b) Data generation

Methods for DNA extraction, amplification and sequencing followed those of Schneider *et al.* (2004a). Sequences of *rbcl* and *trnL-F* regions were generated with published primer pairs (Murakami *et al.* 1999; Trewick *et al.* 2002), whereas the *rps4* and *rps4-trnS* IGS region were sequenced with a novel forward primer (5'-ATG TC[AC] CGT TA[CT] CGA GG[AG] CCT CGT-3') combined with a previously used reverse primer (5'-TAC CGA GGG TTC GAA TC-3'). Sequences were aligned manually using MACCLADE 4.0 (Maddison & Maddison 2000) and ambiguously aligned regions were excluded from all analyses. Gaps were treated as missing data. Indels having a length of at least two base pairs were identified and scored with an assumption of independence for overlapping indels. We compiled two datasets for analysis: (i) a large dataset of nucleotide sequences derived from all four plastid DNA markers for 77 species of asplenoid ferns, including *Diellia*, and (ii) a small dataset of nucleotide sequences derived from the *trnL-trnF* region for 30 species, including all species of *Diellia* and increased sampling of the asplenoid clades related to *Diellia*, as recovered from analyses using the large dataset.

(c) Phylogenetic analyses

PAUP* 4.0b10 (Swofford 2002) was used to conduct maximum-parsimony (MP) analyses, maximum-likelihood (ML) analyses, and corresponding non-parametric bootstrap (BS) analyses using search strategies as described in Schneider *et al.* (2004a). We examined compatibility among the four genes in the large dataset by visually comparing the MP-BS consensus trees from each partition. ML analyses were performed for both datasets with the appropriate model of evolution (GTR + I + Γ) as identified using MODELTEST (Posada & Crandall 1998). Bayesian inference of phylogeny (BY) was performed using MRBAYES 3.0 (Huelsenbeck & Ronquist 2001) with four chains, run for 10^7 generations, keeping only trees generated during stationary generations for use in calculating the Bayesian consensus tree. The evolution of morphological characters and indels was reconstructed with MACCLADE 4.0 applying both ACCTRAN and DELTRAN optimization in a molecular-based MP framework.

(d) Estimating divergence times

A likelihood-ratio test rejected the presence of a molecular clock. Therefore, we used non-parametric rate smoothing (NPRS; Sanderson 2003) to transform the ML phylogram into a chronogram. This was calibrated with the earliest divergence of the genus *Asplenium* (40.44 Myr ago with a 95% confidence interval (CI) of 35.8 to -45 Myr ago), as estimated in Schneider *et al.* (2004b), because no unequivocally assignable fossil is currently available for calibrating the phylogeny of asplenoid ferns. To estimate error based on the uncertainty of the calibration, we calculated 95% CIs using the upper and lower boundary of the CI of the calibration point (Schneider *et al.* 2004b).

3. RESULTS

In all analyses, *Diellia* is resolved as monophyletic and nests within a large derived clade (VIa, figure 1) of *Asplenium* that includes members of the 'black-stemmed' rock spleenworts. Clade VIa was recovered with high BS support (95% or more) and significant BY support ($p \geq 0.95$). MP, ML and BY analyses recovered five strongly supported (100%, $p = 100$) monophyletic groups within the 'black-stemmed' rock spleenwort clade VIa. The *Diellia* subclade (VIa-3) consists solely of species of *Diellia* and is sister to the *A. normale* subclade. The latter subclade includes the Hawaiian endemic *A. hobbeyi* besides the palaeotropical species *A. normale*. The 'black-stemmed' rock spleenworts (VIa) comprise three additional subclades: *A. viride* subclade (VIa-1) including the north temperate *A. viride* Hudson; the *A. trichomanes* subclade (VIa-4) including the *A. trichomanes* complex, along with *A. anceps* Lowe ex Hook. & Grev.; and the *A. monanthes* subclade (VIa-5) consisting mostly of neotropical (occasionally West African) species such as *A. formosum* Willd., *A. monanthes* L. and *A. resiliens* Kunze. The *A. trichomanes* (VIa-4) and *A. monanthes* (VIa-5) subclades were resolved as sisters with high support (BS = 100%). The phylogram for the 'black-stemmed' rock spleenwort clade (VIa) revealed branches of considerable length subtending the *Diellia* (VIa-3) and *A. normale* (VIa-2) subclades. The general topology of the phylogeny external of the 'black-stemmed' rock spleenwort (VIa) clade is consistent with the topology of asplenoid ferns recovered previously and reveals nine distinct major clades (I-IX; figure 1).

Increased taxon sampling in the small dataset (figure 2) provided further insight into the diversification of the 'black-stemmed' rock spleenworts. As found in our analyses of the combined large dataset, both the *A. normale* subclade (VIa-2) and the *Diellia* subclade (VIa-3) terminate long branches. In addition, analyses of the small dataset show that the *A. viride* subclade (VIa-1) terminates a long branch. These long branches contrast sharply with the short branches that interconnect these same subclades. Nucleotide variation was insufficient to resolve the phylogenetic relationships within the *Diellia* clade (VIa-3), whereas we found sufficient variation in both the *A. trichomanes* (VIa-4) and the *A. monanthes* (VIa-5) subclades to support a fully resolved phylogeny to the level of subspecies.

Estimates of divergence times indicated that the *Diellia* lineage is old and has long been separated from other groups of 'black-stemmed' rock spleenworts (*ca.* 24.3 Myr ago, CI = 21.5–27.0). By contrast, extant species of *Diellia* are remarkably young (*ca.* 2 Myr ago, CI = 1.8–2.3), indicating that the lineage has only very recently radiated within

Figure 1. Phylogram based on the large dataset recovered in ML analyses. Branch length corresponds to the number of substitutions per branch as estimated with the ML approach. Thick branches indicate a support of at least $p = 0.95$ in Bayesian analyses and 85% in maximum-parsimony bootstrap analyses. The following major clades are marked: I. *Hymenasplenium*, II. *A. auritum*–*A. juglandifolium* clade, III. *A. cuneifolium*–*A. ruta-muraria* clade, IV. *A. bullatum* clade, V. *A. marinum* clade, VI. Temperate rock spleenworts, VI. *Tarachia*, VII. *Ceterach*–*Phyllitis* clade, IX. *Loxoscaphe*–*Thamnopteris* clade. The clade VIa, ‘black-stemmed’ rock spleenworts, includes five subclades: VIa-1 *A. viride* clade, VIa-2 *A. normale* clade, VIa-3 *Diellia* clade, VIa-4 *A. trichomanes* clade, VIa-5 *A. monanthes* clade.

the Hawaiian islands (figure 3). The sister clades (VIa-2, VIa-4 and VIa-5) show a long history of separation, but evince a steadier accumulation of plastid nucleotide variation.

4. DISCUSSION

Our results support Wagner’s hypothesis of the origin of *Diellia* within the ‘black-stemmed’ rock spleenworts (VIa).

Although analyses of the combined large dataset identify the *A. normale* subclade (VIa-2) as sister to *Diellia* (VIa-3), significant differences in nucleotide substitution and indel structure between these groups indicate that it is unlikely that *A. normale* represents the direct ancestor of *Diellia*. The long period of time following the divergence of *Diellia* from other asplenoid ferns is consistent with the remarkable number of indels found in the non-coding regions of

Figure 2. Phylogram based on the small dataset. ML analyses with midpoint rooting. Branch length corresponds to the number of substitutions per branch as reconstructed with the ML approach. Clade names are as in figure 1. Thick branches have a bootstrap of at least 95%. Vertical bars indicate the occurrence of indels plotted onto this phylogeny using DELTRAN optimization in a maximum-parsimony framework. Distributions are given for each of the five major clades.

plastid DNA markers in *Diellia* as compared to related asplenoid ferns (figure 2).

Much of the confusion regarding the relationship of *Diellia* to closely related asplenoid ferns stems from the unique combination of characters exhibited by this group, namely the co-occurrence of marginal sori and laminae with anastomosing veins. Within asplenoid ferns, marginal sori occur in *Loxoscaphe*, whereas regularly anastomosing venation occurs in the unrelated *Camptosorus* and *Ceterach* lineages. The tendency to focus on, and to account for, two such unusual morphological features may have helped to obscure the many more subtle, but phylogenetically more informative, morphological traits that *Diellia* shares with species of the 'black-stemmed' rock spleenworts such as scale morphology and petiole anatomy. Because continued recognition of *Diellia* as an independent genus would render *Asplenium* paraphyletic, we follow Viane & Reichstein (1991) who transferred all species of *Diellia* to *Asplenium*.

The recovered divergence time estimates suggest separation of the *Diellia* lineage at *ca.* 24.3 Myr ago (CI = 21.5–27.0), but a recent radiation at only *ca.* 2 Myr ago (CI = 1.8–2.3). This estimate fits remarkably well with recent geological evidence concerning the evolution of the Hawaiian-Emperor chain of islands. The evidence suggests that these islands have existed since at least 85 Myr ago and that there was a pronounced lull in new island formation from *ca.* 33 to 23 Myr ago, such that most terrestrial life on the Hawaiian archipelago would have gone extinct because of island subsidence and erosion to sea level (Clague 1996;

Price & Clague 2002). The estimated divergence time of the *Diellia* lineage from the 'black-stemmed' rock spleenworts clade essentially coincides with the estimate of the renewal of Hawaiian terrestrial life at *ca.* 23 Myr ago (Clague 1996; Price & Clague 2002). Thus our data suggest that the ancestor of *Diellia* may have been among the first successful colonists to the newly emerging islands in the early Miocene. The formation of the modern Hawaiian islands starting at *ca.* 5.2 Myr ago precedes the estimated time of the rapid radiation of extant *Diellia* (*ca.* 2 Myr ago) but the estimate is older than the younger major island of Maui (*ca.* 1.3 Myr ago), Lana'i (*ca.* 1.3 Myr ago) and Hawaii (*ca.* 0.43 Myr ago) (Clague 1996). We conclude that (i) the *Diellia* lineage is considerably older than contemporary Hawaiian montane habitats, arriving at the extant islands by hopping down the island chain, (ii) the rapid radiation is probably caused by the development of montane habitats during the formation of islands that grew taller than 1 km (Price & Clague 2002).

Diellia is characterized by several paradoxical features: (i) long genetic isolation versus rapid, recent radiation; (ii) low plastid DNA variation versus high morphological variation; and (iii) substantially increased rate of indel mutation and accumulation versus a more or less constant rate of substitution and accumulation found in sister clades. These features suggest that *Diellia* genetically diverged from other 'black-stem' rock spleenworts long before its current radiation in Hawaii.

Figure 3. Simplified chronogram generated by applying NPRS to the ML phylogeny shown in figure 1. With the exception of clade VI, all major lineages are shown as thickened. The origin of *Diellia* (ca. 24.3 Myr ago) coincides with the renewal of Hawaiian terrestrial life and contrasts with the recent rapid radiation (ca. 2 Myr ago) of the endemic fern genus.

Our evidence suggests that *Diellia* is a palaeo-endemic of the Hawaiian island chain, although it is equally possible that the lineage evolved its distinctive morphology before dispersing to Hawaii, with its apparent endemism resulting from extinction of extra-Hawaiian populations. The present diversity in Hawaii can be attributed to a recent adaptive radiation (figure 3). The recovered pattern is similar to radiations of angiosperms that have occurred in Hawaii and on other geographically isolated oceanic islands. For the ca. 22 Myr time-gap between the origin of *Diellia* and its most recent radiation we assume previous radiation–extinction cycles in relation to island formation and submergence.

As with other endemic Hawaiian plants and animals (Sakai *et al.* 2002), prospects for the successful conservation and continued preservation of the genetic diversity of *Diellia* remain grim (Agurauja & Wood 2002, 2003). It appears that at least one diellioid species, *D. leucostegioides*, has become extinct recently. Such extinction is all the more regrettable given the probable ancestral position of *D. leucostegioides* within the *Diellia* subclade. It is clear, however, that obtaining greater understanding of the origin and evolution of *Diellia* depends greatly on the preservation of the remaining fragments of its diversity. Each species is the product of a unique set of historic events and evolutionary processes.

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