

MOLECULAR PHYLOGENY AND ADAPTIVE RADIATION OF THE ENDEMIC HAWAIIAN *PLANTAGO* SPECIES (PLANTAGINACEAE)¹

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Insular oceanic islands provide excellent opportunities for the study of evolutionary processes and adaptive radiation. The Hawaiian *Plantago* radiation comprises six endemic taxa showing considerable inter- and intraspecific morphological and ecological diversity. The rDNA internal (ITS) and external (ETS) transcribed spacers and two recently described chloroplast spacers, *ndhF-rpl32* and *rpl32-trnL*, were sequenced to study phylogenetic relationships within this morphologically complex group. Phylogenetic analysis provided strong evidence for the monophyly of Hawaiian *Plantago*, suggesting that the lineage arose from a single long-distance dispersal event. Inconsistencies between nuclear and chloroplast phylogenies suggest a history of hybridization. The basal, unresolved dichotomy of the combined phylogeny is consistent with rapid phenotypic diversification of the major lineages early in the history of this group. Speciation has largely occurred allopatrically, with divergence a result of intransland ecological shifts between bog and woodland habitats and interisland dispersal events. Most interisland colonizations were from older to younger islands with initial colonization of Kaua'i. In our analysis, *P. pachyphylla* is paraphyletic and taxonomic separation of the distinct morphotypes of this species appears justified. Furthermore, the apparent hybrid ancestry and unique morphology and habitat of the endangered *P. princeps* var. *longibracteata* support its recognition at the specific rank.

Key words: adaptive radiation; biogeography; Hawaiian Islands; hybridization; *ndhF-rpl32*; phylogeny; Plantaginaceae; *Plantago*; *rpl32-trnL*.

Insular oceanic islands offer isolation from source populations and a close association of a diversity of habitats. The evolutionary consequences are rapid speciation events often coupled with remarkable examples of adaptive radiation (Darwin, 1859). The Hawaiian Archipelago encompasses an ideal system in which to study these phenomena. Islands in the archipelago were formed chronologically with the younger, current high islands (Kaua'i, O'ahu, Moloka'i, Maui, Lāna'i, Kaho'olawe, and Hawai'i) occupying the southeast portion of the chain (Carson and Clague, 1995; Funk and Wagner, 1995). The archipelago is also the most isolated large group of islands in the world (ca. 3800 km from the nearest continental migrant source), which has resulted in infrequent colonization and thus an increased chance that the patterns observed in endemic lineages can be attributed to single colonization events (Fosberg, 1948; Carlquist, 1980). Hawaiian lineages are often further characterized by rapid diversification enhanced by reduced

competition and a diversity of habitats generally in close proximity to one another (Carlquist, 1974; Price and Wagner, 2004). These characteristics allow evolutionary patterns to be more easily discerned, making Hawaiian lineages important subjects of evolutionary study. Despite this, there have been relatively few studies of phylogenetic and potentially adaptive radiations in Hawaiian plant groups (Helenurm and Ganders, 1985; Baldwin et al., 1991; Givnish et al., 1995; Ballard and Sytsma, 2000; Carlquist et al., 2003; Lindqvist et al., 2003; Nepokroeff et al., 2003; Geiger and Ranker, 2005; Howarth and Baum, 2005; Sakai et al., 2006). This situation is no doubt in part due to the recent derivation of these groups, which has made it difficult to reconstruct their evolutionary histories with commonly used molecular phylogenetic markers, and as a result, has left many Hawaiian plant lineages in need of phylogenetic study.

To further our understanding of adaptive radiation and island evolution in a Hawaiian plant group, we have focused on the endemic Hawaiian *Plantago* L. species. *Plantago* is a large genus (ca. 200–260 species) with a worldwide distribution. Morphological classification groups the Hawaiian species into section *Plantago* (ca. 42 species), which includes many oceanic island species in addition to North American and Old World taxa (Rahn, 1996). Similar to other Hawaiian plant groups, the Hawaiian *Plantago* species occur in a variety of habitats such as bogs, wet ridges tops, subalpine shrubland, mesic forest, rainforest, and waterfalls and have diverged in growth form (including woodiness), leaf morphology, and reproductive features. As such, conclusions drawn from this study may have relevance to similar groups. Nevertheless, Hawaiian *Plantago* is understudied and relatively poorly known. The most recent treatment of the Hawaiian flora recognizes only three endemic species and four varieties of *Plantago* [*P. pachyphylla* A. Gray, *P. hawaiiensis* (A. Gray) Pilg., and *P. princeps* Cham. & Schltdl. var. *anomala* Rock, *P. princeps* Cham. & Schltdl. var. *laxifolia* A. Gray, *P. princeps* Cham. & Schltdl. var. *longibracteata* H. Mann, and *P. princeps* Cham. & Schltdl. var. *princeps*] (Wagner et al., 1990). However, these species' considerable inter- and

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intraspecific morphological and ecological diversity has led to broad disagreement regarding the number of taxa that should be recognized and their relationships to one another (Gray, 1862; Hillebrand, 1888; Rock, 1920; Pilger, 1923, 1936, 1937; Skottsberg, 1944; Wagner et al., 1990). Further complicating species delimitations in the group is the apparent, frequent hybridization in areas of sympatry where morphological differences between taxa and morphotypes can often be obscured. The substantial morphological diversity encompassed by populations of *P. pachyphylla* and *P. princeps*, in particular, have led researchers to suggest that these species are in fact species complexes (Wagner et al., 1990). Morphotypes within these species can be differentiated by habitat; growth form; leaf arrangement, attachment, and pubescence; and the position of the flower relative to the spike (Table 1). Greenhouse experiments carried out in conjunction with this study indicate that these differences are genetically based and not simply from phenotypic plasticity (Dunbar-Co et al., in press). Within Hawaiian *Plantago*, growth form appears to be correlated with habitat; woody morphotypes are found largely in woodland habitats (includes forest and shrubland habitats), whereas herbs are primarily restricted to open bogs or boglike habitats such as wet ridge tops. Morphological and ecological characteristics provide support for loosely subdividing Hawaiian *Plantago* into 13 distinct morphotypes (Table 1).

Here, we conducted a molecular phylogenetic analysis to delimit species, elucidate relationships among them, and examine evolutionary patterns in this poorly known group using DNA sequences of the ribosomal DNA internal (ITS) and external (ETS) transcribed spacers in combination with two rapidly evolving spacer regions from the chloroplast genome, *ndhF-rpl32* and *rpl32-trnL*. The primary objectives of this study were to (1) present the first phylogeny of the Hawaiian *Plantago* radiation, (2) assess the monophyly of the group, (3) elucidate relationships among Hawaiian *Plantago* taxa and

morphotypes, including an assessment of possible hybridization between species, (4) determine modes of speciation, and (5) investigate the historical biogeography of the group.

MATERIALS AND METHODS

Taxon sampling and DNA extraction—The abundant intraspecific variation and the lack of a full systematic treatment prompted us to collect leaf material from all known extant, morphologically and geographically distinct populations. Within islands, samples were collected from different volcanoes to give age structure within islands (e.g., West Maui vs. East Maui, Wai'anae vs. Ko'olau). A total of 21 Hawaiian *Plantago* individuals, including multiple accessions of all currently recognized taxa, were collected from every island and included for analysis (Appendix 1). To assess intrapopulation variation, two or three individuals were sampled from every population. *Plantago macrocarpa* Cham. & Schltdl., *P. rapensis* Pilg., *P. rupicola* Pilg., and *P. spathulata* Hook.f. were used as outgroup taxa for this study based on DNA sequence analysis of section *Plantago* that included the three Hawaiian species (R. K. Hoggard [University of Oklahoma], N. Rønsted [Kew], S. Dunbar-Co [Univ. of Hawai'i], and P. J. Kores and W. J. Elisens [Univ. of Oklahoma], unpublished data).

Total genomic DNA was extracted from fresh leaf tissue using a slightly modified version of the CTAB extraction protocol (Doyle and Doyle, 1987) or from silica-gel-dried tissue using the DNeasy Plant Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Samples were accessioned into the Hawaiian Plant DNA Library (Morden et al., 1996; Randell and Morden, 1999).

Molecular markers—Nuclear and chloroplast DNA gene regions were obtained for phylogenetic analysis. The internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions of the nuclear ribosomal DNA have both been shown to provide species-level phylogenetic signal (Baldwin et al., 1995; Baldwin and Markos, 1998; Linder et al., 2000; Andreasen and Baldwin, 2001; Markos and Baldwin, 2001; Nepokroeff et al., 2003; Timme et al., 2007). Two intergenic spacers from the small single-copy region of the chloroplast genome (*ndhF-rpl32* and *rpl32-trnL*) were also sequenced having recently yielded appropriate amounts of variation for species and even population-level phylogenetic studies (Shaw et al., 2007). Including gene regions from the nuclear (biparentally inherited) and chloroplast (uniparentally inherited) genomes

TABLE 1. Morphological and ecological variation in Hawaiian *Plantago*. H, Hawai'i; K, Kaua'i; eM, East Maui; wM, West Maui; Mo, Moloka'i; eO, eastern O'ahu; wO, western O'ahu.

Taxon/Morphotype	Island(s)	Habitat	Growth form ^b	Leaf pubescence	Leaf arrangement ^c	Leaf attachment	Floral position to spike
<i>P. hawaiiensis</i> ^a	H	Subalpine shrubland	Herb	Glabrous	Basal	Petiolate	Ascending
<i>P. hawaiiensis</i> ^a and <i>P. pachyphylla</i>	H, eM, wM, Mo	Bog	Herb, robust	Glabrous or sparse abaxial tomentum	Equitant	Sessile	Ascending
<i>P. pachyphylla</i>	wM	Bog	Herb, minute	Glabrous	Rosette	Sessile	Ascending
<i>P. pachyphylla</i>	wM	Rainforest	Herb, robust	Dense abaxial tomentum	Basal	Petiolate	Ascending
<i>P. pachyphylla</i>	Mo	Wet ridge	Herb, robust	Dense adaxial and abaxial tomentum	Basal	Sessile	Ascending
<i>P. pachyphylla</i>	K, eO	Bog/Wet ridge	Herb	Glabrous	Rosette	Sessile	Ascending
<i>P. pachyphylla</i>	K	Bog/Wet ridge	Herb, minute	Glabrous	Rosette	Sessile	Ascending
<i>P. pachyphylla</i>	K	Bog/Wet ridge	Herb	Dense adaxial and abaxial tomentum	Rosette	Sessile	Ascending
<i>P. pachyphylla</i>	K	Bog	Herb, minute	Glabrous or dense adaxial tomentum	Rosette	Sessile	Ascending
<i>P. princeps</i> var. <i>anomala</i> ^a	K	Rainforest	Shrub, erect	Glabrous	Whorled	Sessile	Perpendicular
<i>P. princeps</i> var. <i>laxifolia</i> ^a	eM, wM	Rainforest	Shrub, erect	Glabrous	Whorled	Petiolate	Perpendicular
<i>P. princeps</i> var. <i>longibracteata</i> ^a	K	Waterfall	Subshrub, prostrate	Glabrous	Whorled	Petiolate	Ascending
<i>P. princeps</i> var. <i>princeps</i> ^a	eO, wO	Mesic forest	Shrub, erect	Glabrous	Whorled	Petiolate	Perpendicular

^a Federally listed endangered species

^b Robust = plants greater than 8 inches in height; minute = plants less than one inch in height; erect = vertical; prostrate = lying on the ground.

^c Basal = leaves that arise from the base of the stem and are spirally arranged; equitant = leaves overlapping in two ranks; rosette = dense radiating cluster of leaves at ground level, the stem very short or lacking; whorled = three or more leaves arising from a node at the tips of stems.

allowed us to assess discordance among the respective phylogenies, giving insight regarding the influence of hybridization on the Hawaiian *Plantago* species (Rieseberg and Soltis, 1991; Baldwin, 1997; Sang and Zhong, 2000).

Amplification and DNA sequencing—The four gene regions were amplified and sequenced using standard primers found in the literature (White et al., 1990; Downie and Katz-Downie, 1996; Baldwin and Markos, 1998; Shaw et al., 2007). ITS amplifications used the following polymerase chain reaction (PCR) conditions: 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, final extension of 72°C for 7 min. ETS amplification conditions differed by having a 61°C annealing temperature and used a newly developed 5' primer (PlanF: GTACCTGGGAAGGACACCAA) located approximately 750 bp upstream of the 18S gene. Chloroplast gene amplifications used the following cycling conditions: 95°C for 2 min, 30 cycles of 95°C for 30 s, 50°C for 1 min followed by a ramp of 0.3°C/s to 65°C, 65°C for 4 min, final extension of 65°C for 5 min.

All amplifications were carried out using a MJR thermal cycler (Waltham, Massachusetts, USA) for 25- μ L reactions containing 1.25 units *Taq* DNA Polymerase (Roche Applied Science, Mannheim, GER) or 2.5 units Proofstart DNA Polymerase (Qiagen, Valencia, California, USA), 0.2 mM of dNTPs in an equimolar ratio, 10 \times buffer containing 1.5 mM MgCl₂, 0.5–2 μ M each primer, and 20–30 ng genomic DNA. Dimethyl sulfoxide (4%) was added to reactions of species that routinely had difficulty amplifying. Polymerase chain reaction products were visualized by agarose gel electrophoresis and cleaned using either a PCR Purification kit (Qiagen) or treatment with shrimp alkaline phosphatase and exonuclease I (ExoSAP; USB Corp., Cleveland, Ohio, USA).

Samples were bidirectionally sequenced at the University of Hawai'i's AS-GPB Sequencing Facility (<http://cgpr.hawaii.edu/>) using BigDye Terminator chemistry (Applied Biosystems, Foster City, California, USA) and visualized on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems). A few amplified products of ITS that did not directly sequence well were cloned using the Invitrogen TOPO TA Cloning Kit for Sequencing (Carlsbad, California, USA), and 3–5 clones of ~1.2 kb were sequenced. Cloned sequences from an individual were identical.

Sequence alignment and phylogenetic analysis—Contiguous sequences were constructed and edited for all ITS, ETS, *ndhF-rpl32*, and *rpl32-trnL* sequences using BioEdit version 7.0.5.3 (Hall, 1999). Because of low sequence polymorphism, sequences for each region were easily aligned manually in MacClade version 4.08 (Maddison and Maddison, 2005). Individuals within a population with identical sequences for all four gene regions were represented by a single individual in the analysis. Due to difficulty sequencing through A repeats for one individual (*P. pachyphylla*, Hanawi) in *rpl32-trnL*, an ~180-bp region of the spacer was input as missing data. The individual was retained in the data set because of low sequence polymorphism in this region and because its inclusion did not appear to influence the analysis.

Indels were coded as missing data in all presented analyses. However, analyses were conducted with and without indels coded as presence/absence characters for each data set using a simple gap coding strategy (Simmons and Ochoterena, 2000). Gaps consisting of A and T repeats were not coded given that homology in these areas appeared weak. The data were separated into two major partitions, nuclear (ITS, ETS) and chloroplast (*ndhF-rpl32*, *rpl32-trnL*). The data partitions were compared using the incongruence length difference (ILD) test of Farris et al. (1994), implemented as the partition homogeneity test in PAUP* version 4.0b10 (Swofford, 2002). For each test, 1000 replicates were performed using heuristic searches, tree-bisection-reconnection (TBR) branch swapping with random addition for three replicates, nchuck = 2, and chuck-score = 1. The ILD tests were done with (1) all individuals included, (2) one discordant individual removed at a time in successive analyses, and (3) groups of discordant individuals removed.

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods were used to estimate phylogenies for all three data sets (nuclear, chloroplast and combined). Maximum parsimony and ML searches were conducted using PAUP* 4.0b10 (Swofford, 2002), and Bayesian analyses were performed in MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001). In all MP analyses, heuristic searches were done using a starting tree built from stepwise addition with 1000 random addition replicates and TBR branch swapping. Bootstrap analyses based on 1000 replicates with 10 random additions per replicate were used to assess confidence in clades. Maximum likelihood trees were estimated from heuristic searches started via stepwise addition with 100 random addition replicates and TBR branch swapping. Branch support for ML trees was estimated from 500 bootstrap replicates with five random addition replicates per bootstrap. Evolutionary models were selected by Akaike information criterion

(AIC; Akaike, 1974) using ModelTest version 3.7 (Posada and Crandall, 1998). The models for separate and combined data partitions are as follows: nuclear, TrN+G; chloroplast, TIM+G; combined data set, TIM+I+G. For Bayesian analyses, a mixed model analysis was run so that each gene could evolve under its own best-fit model. Bayesian analyses were run using four incrementally heated chains for 1 000 000 generations and a sampling frequency of 100 generations. Tree samples and parameter estimates from the first 2000 trees were designated as the burn-in period and discarded.

RESULTS

Coded indels—The majority of the informative gaps were not homoplastic when mapped on to the substitution-based gene trees; however, they did not contribute a significant amount of phylogenetic information to any of the data sets. Instead, their inclusion reduced resolution, bootstrap support for clades, and CI levels in the combined analysis. As such, coded indel characters were omitted from all subsequent analyses. The number of coded indels for each gene region and partition are given in Table 2.

Separate partition analyses—All the gene regions were initially analyzed separately. Numbers of individuals, parsimony informative characters (PICs), and alignment lengths for each region and partition are shown in Table 2. The different gene regions did not vary substantially in the amount of phylogenetic information they contained. Similarly, the nuclear and chloroplast gene partitions did not differ in total number of PICs (41 vs. 42, respectively; Table 2), though more PICs differentiated between members of the ingroup in the chloroplast partition than in the nuclear partition.

Nuclear partition—The alignment of ITS and ETS sequences resulted in 1241 characters; 41 were variable and parsimony informative, and 61 were variable but parsimony uninformative (i.e., autapomorphic; Table 2). The 1000 MP heuristic searches resulted in three trees with length 115 and CI = 0.90. The 100 ML searches using the TrN+G base substitution model produced one tree with score $-\ln L = 2409.24$ and Bayesian analysis resulted in a single consensus tree with score $-\ln L = 2439.92$. Because the three different optimality criteria (MP, ML, and Bayesian) produced phylogenies that were largely concordant, only the ML tree reconstructions are shown here (Fig. 1). Clade support within trees is based on bootstrap values and posterior probabilities where high support is >90%, moderate support 70–90%, and low support <70%.

The nuclear data set strongly supports the monophyly of Hawaiian *Plantago* and moderately supports a sister relationship with the western North American species, *P. macrocarpa* (Bayesian support is high) (Fig. 1). *Plantago pachyphylla* is paraphyletic as evidenced by the split between the Kaua'i vs. the O'ahu and Maui Nui morphotypes. Despite high branch support for these groups, relationships within them were unresolved (support values $\leq 50\%$; Fig. 1). Monophyly of the woody shrub *P. princeps* varieties is weakly supported by Bayesian analysis. In general, the three major nrDNA lineages most closely correspond with morphology and habitat type: minute, bog forms from Kaua'i; woody *P. princeps* varieties; and herbaceous bog and woodland forms from the younger islands (Fig. 1).

Chloroplast partition—Concatenated sequences of *ndhF-rpl32* and *rpl32-trnL* resulted in 1607 characters; 42 were parsimony informative, while 68 were variable but parsimony

TABLE 2. Sequence characteristics for each gene partition.

Partition	N ^a	Length (bp)	Aligned length (bp)	Parsimony informative characters	Variable, parsimony uninformative characters	Coded indels
ITS	25	664–675	548	22	32	5
ETS	25	763–775	693	19	29	9
<i>ndhF-rpl32</i>	25	776–844	801	25	30	22
<i>rpl32-trnL</i>	24	790–842	806	17	38	13
Combined nuclear regions	25	n/a	1241	41	61	14
Combined plastid regions	25	n/a	1607	42	68	35
Combined all regions	20	n/a	2848	77	134	49

^aThe number of individuals in each data set after removing those with identical sequences within a population. Five discordant individuals were also removed from the combined analysis of all regions.

uninformative (Table 2). The MP heuristic searches recovered 10 equally parsimonious trees of length 123 and CI = 0.94. The 100 ML searches under the TIM+G model yielded a single tree of score $-\ln L = 2856.62$ and the Bayesian consensus tree had a best score of $-\ln L = 2885.48$. As with the nuclear data, the three analyses produced trees that were largely concordant, and only the ML tree reconstructions are shown (Fig. 1). Monophyly of Hawaiian *Plantago* is also strongly supported by the chloroplast data set, but in contrast to the nuclear regions, tree reconstructions of the chloroplast partition indicate a sister relationship between the Hawaiian and Rapan taxa; a relationship previously hypothesized by R. K. Hoggard [University of Oklahoma], N. Rønsted [Kew], S. Dunbar-Co [Univ. of Hawai'i], and P. J. Kores and W.J. Elisens [Univ. of Oklahoma] (unpublished data).

Within the ingroup, there is evidence of two primary clades, though the relationship of these clades to one another is unresolved (Fig. 1). The first clade contains all of the collections from Kaua'i, except *P. princeps* var. *anomala*, and the sole O'ahu *P. pachyphylla* morphotype. *Plantago princeps* var. *longibracteata* from 'Ili'ili'ula and *P. pachyphylla* from Sincok Bog are sister to the rest of the Kaua'i individuals, including a second collection of *P. princeps* var. *longibracteata* from Blue Hole, Kaua'i. The second clade consists of individuals of *P. princeps* from O'ahu and Maui, *P. pachyphylla* from Moloka'i and Maui, and *P. hawaiiensis* from Hawai'i. Resolution in this second clade is generally low, and branch support for relationships is weak with the exception of well-supported relationships between the two *P. hawaiiensis* collections, the East Maui collections of *P. princeps* var. *laxifolia* (Kipahulu) and *P. pachyphylla* (Hanawi), and the diverse *P. pachyphylla* morphotypes from Moloka'i (in Bayesian analysis). Despite low resolution of the chloroplast phylogeny, these data strongly support parphyly of *P. pachyphylla* and *P. princeps*. In contrast to the nuclear data set, the groupings presented here correspond geographically, where individuals are largely affiliated by region (i.e., island or volcano within island), generally irrespective of taxonomic affiliation, morphology, or habitat type.

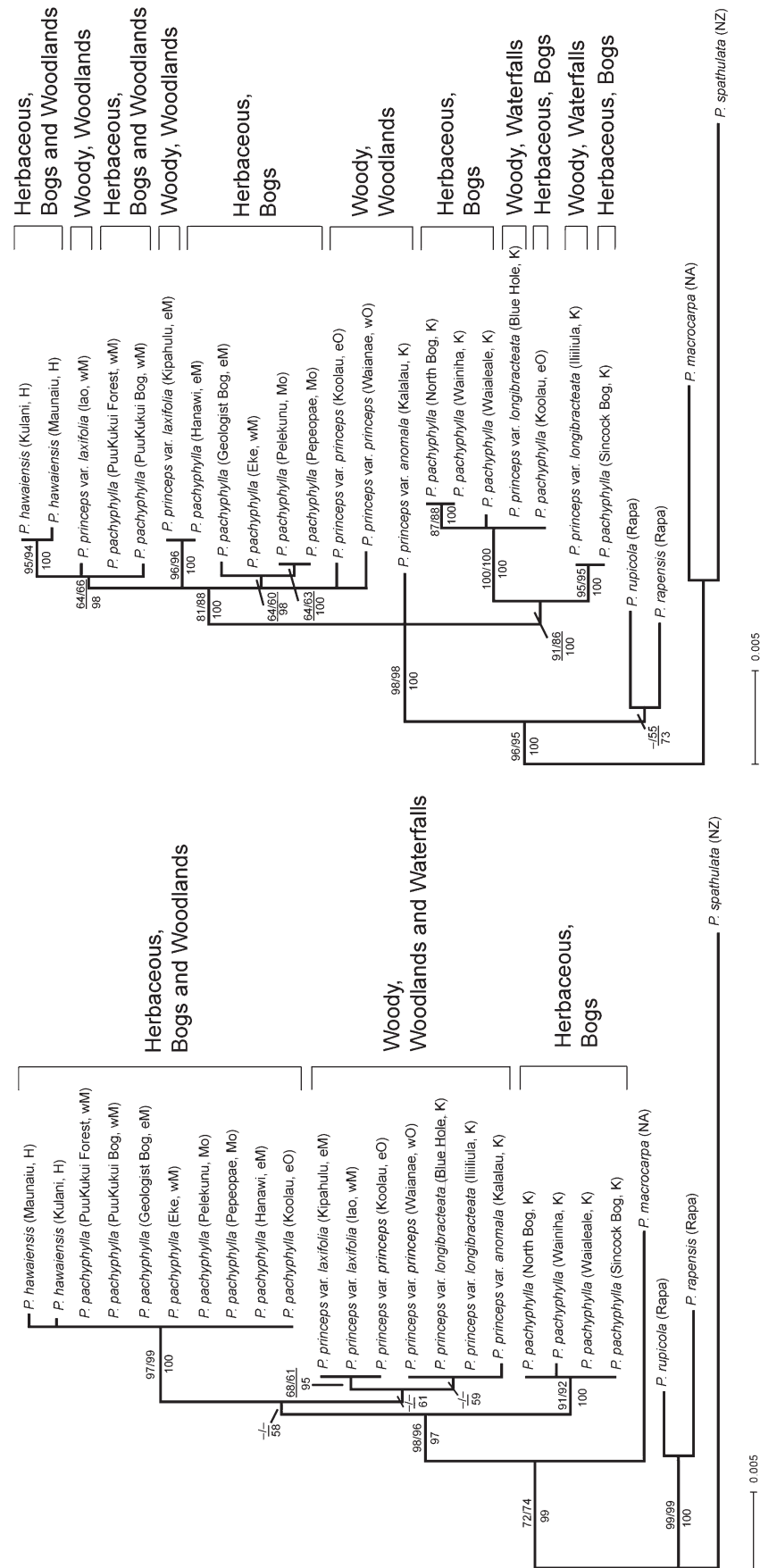
Combined analyses—Incongruence length difference tests for the combined matrix revealed that there was a highly sig-

nificant level of incongruence ($P = 0.001$) between the nuclear and chloroplast gene partitions. Closer examination revealed that five individuals representing three taxa were primarily responsible for the discordance between data partitions, *P. princeps* var. *longibracteata* from Blue Hole and 'Ili'ili'ula on Kaua'i, *P. princeps* var. *laxifolia* from 'Iao and Kipahulu on Maui, and *P. pachyphylla* from O'ahu (Fig. 1). When sequences of all five of these individuals were removed from analyses, the gene partitions were no longer significantly discordant ($P > 0.05$).

Combining the gene partitions resulted in a better-resolved tree with higher branch support for clades (Fig. 2) than either of the single gene partition analyses. The combined matrix consisted of 2848 characters, 77 of which were parsimony informative (Table 2). The 1000 MP searches resulted in a single best tree of length = 240 and CI = 0.90. Maximum likelihood searches found one tree of score $-\ln L = 5433.38$, and Bayesian searches gave a consensus tree of score $-\ln L = 5450.71$. Maximum parsimony, ML, and Bayesian analyses all produced identical phylogenies, and the Bayesian tree is shown here (Fig. 2). As in the separate partition analyses, there is strong support for the monophyly of Hawaiian *Plantago* (Fig. 2). Similar to the chloroplast data, a sister relationship between the Hawaiian and Rapan taxa is supported, although ML support of this relationship is weak.

The combined data similarly support the parphyly of *P. pachyphylla* and the existence of two major clades among the Hawaiian species (Fig. 2). The first group consists of herbaceous, bog *P. pachyphylla* morphotypes from Kaua'i, which is well-resolved with high branch support for clades. In this clade, *P. pachyphylla* from Sincok Bog is basal, while the more recently derived Kaua'i morphotypes are from Wainiha and North Bog that occur in the isolated, northern regions of the main Kaua'i bog system (Alaka'i). The second clade consists of *P. princeps*, *P. pachyphylla*, and *P. hawaiiensis* morphotypes from all of the islands. In this clade, *P. princeps* var. *anomala* from Kaua'i is basal followed by *P. princeps* varieties from western (Wai'anae) and eastern O'ahu (Ko'olau), respectively. The most derived group in the lineage is a well-supported, albeit poorly resolved, clade containing *P. pachyphylla* and *P. hawaiiensis* morphotypes from the younger islands of Moloka'i, Maui, and Hawai'i.

Fig. 1. Phylogram trees generated by 100 heuristic maximum likelihood searches of the nuclear (ITS and ETS) and chloroplast (*rpl32-trnL* and *ndhF-rpl32*) data partitions for Hawaiian *Plantago*. Above branches are bootstrap values (MP/ML) and below branches are Bayesian posterior probability scores. Log-likelihood scores: nuclear = 2409.24 and chloroplast = 2856.62 (see Methods and Materials for individual data set models). Refer to Table 1 for island abbreviations. NA, North America; NZ, New Zealand. Note: Woodlands refers to forests and shrublands, and bogs include wet ridge tops.



DISCUSSION

Monophyly of Hawaiian *Plantago*—The affinities of Hawaiian *Plantago* have long been debated (Rock, 1920; Wagner et al., 1990; R. K. Hoggard [University of Oklahoma], N. Rønsted [Kew], S. Dunbar-Co [Univ. of Hawai'i] and P. J. Kores and W. J. Elisens [Univ. of Oklahoma], unpublished data). Based largely on an apparently erroneous chromosome count by Skottsberg (1955) of $2N = 12$ for *P. princeps*, Wagner et al. (1990) hypothesized that Hawaiian *Plantago* may have arisen from two separate dispersal events, one giving rise to the herbaceous *P. pachyphylla* and *P. hawaiiensis* forms ($2N = 24$) and the other to the woody *P. princeps* complex. However, more recent examinations of chromosome number in the group have not been able to replicate Skottsberg's count, and in fact chromosome counts taken for all species and many populations of Hawaiian *Plantago* indicate a single tetraploid number of $2N = 4x = 24$ (Moore, 1973; Kiehn, 2005; S. Dunbar-Co and G. D. Carr [Oregon State University], unpublished data). Data presented here confirm that Hawaiian *Plantago* is monophyletic as evidenced by strong branch support values uniting the Hawaiian taxa relative to the outgroup taxa in all data sets (nuclear, chloroplast, and combined). The sister group to the Hawaiian taxa, on the other hand, is not well resolved. Our nuclear data suggest that the western North American species *P. macrocarpa* is the closest living relative of the Hawaiian taxa, though the chloroplast and combined data indicate a sister relationship with the Rapan taxa, *P. rapensis* and *P. rupicola*. The coastal distribution of *P. macrocarpa*, shorter distance separating Hawai'i and North America vs. Hawai'i and Rapa, and larger size of North America as a source area relative to Rapa, lend support to its sister relationship with the Hawaiian taxa. Additionally, American origins have been found for many Hawaiian plant lineages (Fosberg, 1948; Baldwin et al., 1991; Howarth et al., 1997; Vargas et al., 1998; Ballard and Sytsma, 2000; Lindqvist et al., 2003; Eggens et al., 2007). Alternatively, based largely on morphology, affinities between New Zealand and the Hawaiian taxa have been hypothesized (Rock, 1920; Rahn, 1996; R. K. Hoggard [University of Oklahoma], N. Rønsted [Kew], S. Dunbar-Co [Univ. of Hawai'i] and P. J. Kores and W. J. Elisens [Univ. of Oklahoma], unpublished data), and a possible dispersal pathway to Hawai'i could have been from New Zealand via the remote island of Rapa. This dispersal pattern is very similar to that of other Hawaiian plant groups such as *Metrosideros* (Wright et al., 2001), *Bidens* (Ganders et al., 2000), *Pittosporum* (Gemmell et al., 2002), *Tetraplasandra* (Costello and Motley, 2001), and *Cyrtandra* (Cronk et al., 2005) where close relationships between these Hawaiian groups and their Polynesian relatives have been found. Because the data presented here do not fully resolve the relationship between the Hawaiian taxa and these outgroup species, further investigation is necessary to confirm the sister group of Hawaiian *Plantago*.

Island biogeography of Hawaiian *Plantago*—A common biogeographical pattern in Hawaiian lineages is initial colonization of the oldest high island, Kaua'i, followed by dispersal and evolutionary diversification on each new volcano as it became available for colonization (reviewed in Funk and Wagner, 1995). Overall, Hawaiian *Plantago* appears to follow this pattern of progressive, oldest to youngest, island colonization and the combined phylogenetic data unequivocally indicate a Kaua'i ancestor for the lineage (Figs. 2 and 3). Following establishment on Kaua'i, there was dispersal to and subsequent diversi-

fication on the next oldest volcanoes, Wai'anae on western O'ahu (3.7 My) and Ko'olau on eastern O'ahu (2.6 My), respectively. From Ko'olau, *Plantago* dispersed to one of the younger islands of Moloka'i, Maui, or Hawai'i and speciated; however, because relationships among the most recently derived *P. pachyphylla* and *P. hawaiiensis* morphotypes are unresolved, the dispersal pattern on these younger islands is ambiguous. It is most likely that dispersal proceeded from Ko'olau to Moloka'i or Maui and, finally, to Hawai'i, given that land bridges once connected Ko'olau to western Moloka'i and all of the volcanoes comprising the Maui Nui complex (western and eastern Moloka'i, West and East Maui, Lāna'i, and Kaho'olawe) to one another (Carson and Clague, 1995; Juvik and Juvik, 1998). However, it is possible that *Plantago* dispersed from Ko'olau directly to the island of Hawai'i, in which case instances of back dispersal from Hawai'i to Maui Nui cannot be discounted.

Relationships and ecological shifts in Hawaiian *Plantago*—On the basis of the combined analysis (excluding individuals of *P. princeps* var. *longibracteata*, *P. princeps* var. *laxifolia*, and *P. pachyphylla* from O'ahu), there is evidence of a primary division within the Hawaiian *Plantago* radiation (Fig. 2). This split separates the herbaceous, bog forms on Kaua'i from the woody and herbaceous forms occurring in woodlands and bogs on all of the islands. At the base of this latter clade are woody *P. princeps* varieties from Kaua'i and O'ahu. Carlquist (1970) hypothesized that woodiness in Hawaiian *Plantago* was a derived trait based on the lack of rays in the secondary xylem and phloem in these taxa. More recent work using molecular data on groups in Hawai'i and other island systems have confirmed that woodiness is often derived from herbaceous ancestors (e.g., the silversword alliance, Baldwin and Robichaux, 1995; *Echium*, Böhle et al., 1996; *Pericallis*, Panero et al., 1999; Hawaiian *Viola*, Ballard and Sytsma, 2000; and Hawaiian *Silene*, Eggens et al., 2007), although woodiness in Hawaiian groups such as *Cyanea* (Givnish et al., 1995) and *Psychotria* (Nepokroeff et al., 2003) has been retained from woody colonizing ancestors. In Hawaiian *Plantago*, the nuclear and chloroplast phylogenies suggest that woodiness was likely secondarily derived from the herbaceous growth form. Therefore, despite the fact that the combined data set does not resolve the base of the Hawaiian lineage, it is most likely that the ancestral morphotype within the radiation is an herbaceous *P. pachyphylla* type from Kaua'i. Additionally, the fact that the herbaceous growth form of these morphotypes is shared with most of the outgroup taxa (*P. rupicola* is woody) and that these morphotypes occur on the oldest high island (Kaua'i) further support that the initial *Plantago* colonist was likely most closely related to a herbaceous, bog *P. pachyphylla* type from Kaua'i.

The data suggest that following establishment of Kaua'i bogs by the herbaceous form there was movement out of the bogs and into neighboring woodland habitats. This habitat shift was seemingly accompanied by the development of woodiness and the derivation of the woody, Kaua'i endemic, *P. princeps* var. *anomala*. The combined data indicate that this taxon or its ancestor then dispersed to woodland habitats on the next younger volcanoes of Wai'anae, then Ko'olau and gave rise to *P. princeps* var. *princeps*. There seems to have then been a reversion back to the herbaceous growth form in the most recently derived clade of Hawaiian *Plantago*, which consists of herbaceous morphotypes from the younger islands of Moloka'i, Maui, and

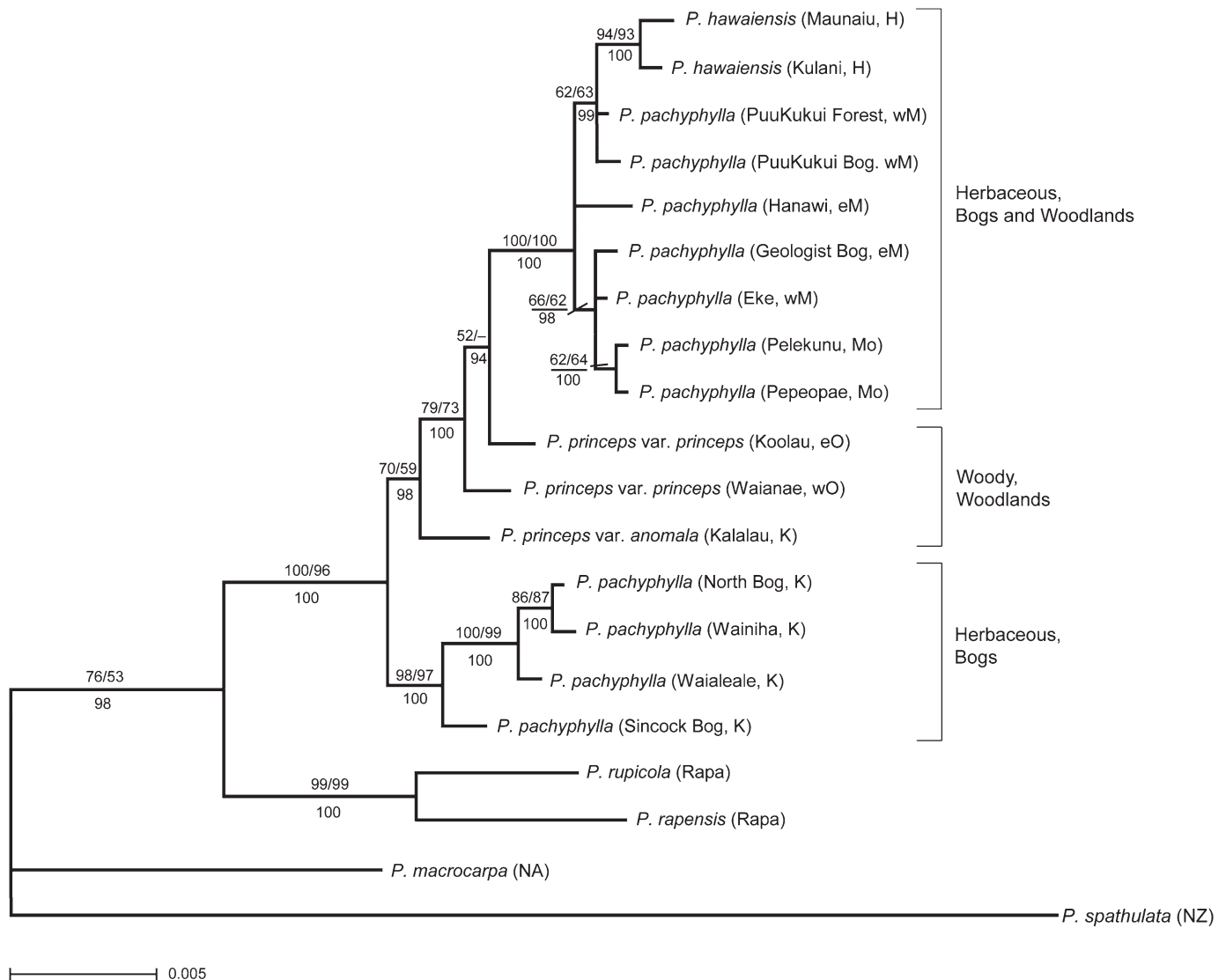


Fig. 2. Bayesian consensus tree for Hawaiian *Plantago* of the combined data set (ITS, ETS, *rpl32-trnL*, and *ndhF-rpl32*) run for 1 million generations with model TIM+I+G and score $-\ln L = 5450.71$. Above branches are bootstrap values (MP/ML) and below branches are Bayesian posterior probability scores. Refer to Table 1 and Fig. 1 for location abbreviations. Note: Woodlands refers to forests and shrublands, and bogs include wet ridge tops.

Hawai'i. This shift in growth form may have been mediated by the availability of bog habitats on these younger islands. Unlike the herbaceous forms on Kaua'i, a few of these more recently derived herbaceous morphotypes, such as *P. pachyphylla* from Pu'u Kukui forest and *P. hawaiiensis* from Maunaiu (Table 1), occur largely in woodland habitats, a condition they may have retained from their woody progenitors. Despite differences in habitat, these later-derived, herbaceous forms represent a well-supported clade (Fig. 2); however, relationships among them are not clearly resolved, a result likely due to the recent and rapid diversification of these forms (cf. Baldwin et al., 1990; cf. Baldwin and Sanderson, 1998). Despite this, within the clade there is strong support that single dispersal events gave rise to the diverse Moloka'i *P. pachyphylla* forms (low support based on MP and ML) and *P. hawaiiensis* forms on the island of Hawai'i. The Maui forms of *P. pachyphylla* do not form a monophyletic group and their relationship to both the Moloka'i and Hawai'i morphotypes is not clear.

Overall, the data suggest a relationship between growth form and habitat type in Hawaiian *Plantago* with herbaceous *P. pachyphylla* and *P. hawaiiensis* forms restricted to open bogs and wet ridge tops, while woody *P. princeps* varieties are primarily found in woodland habitats (Fig. 4). Habitat shifts between bog and woodland habitats, with accompanying changes in growth form and secondary structure, appear to be a major driver of speciation in intransland species radiations in Hawaiian *Plantago*, which is a common trend in Hawaiian plant adaptive radiations (Carlquist, 1974; Funk and Wagner, 1995; Price and Wagner, 2004). In the case of interisland dispersal, it is probable that isolation is great enough to allow for diversification without habitat shifts, and the data presented here do not refute this hypothesis.

Hybridization within Hawaiian *Plantago*—Unlike the other Hawaiian *Plantago* morphotypes, individuals of *P. princeps* var. *longibracteata*, *P. princeps* var. *laxifolia*, and *P. pachyphylla*

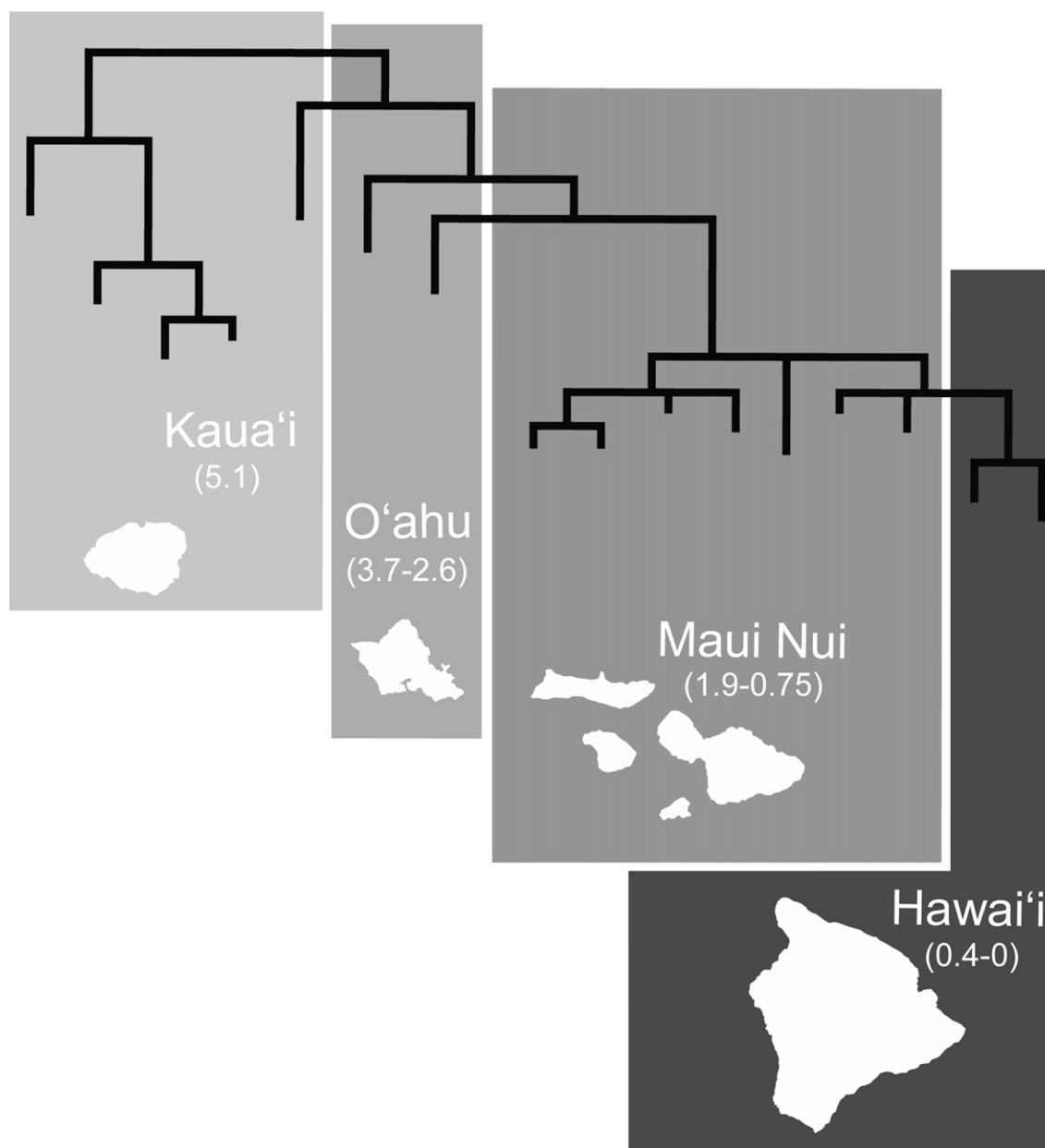


Fig. 3. Biogeographic distribution of Hawaiian *Plantago* morphotypes included in the combined analysis. In parentheses is island age in millions of years.

(O'ahu) demonstrated incongruence among the nuclear and chloroplast gene trees. This evidence, combined with the high interfertility, wind pollination, and largely protogynous breeding system of Hawaiian *Plantago* taxa (Rahn, 1996; S. Dunbar-Co, personal observation), strongly suggest hybridization among these species.

Plantago princeps var. *longibracteata* is a woody, prostrate subshrub whose admixture of morphological traits and novel riparian habitat have led researchers in the past to postulate its hybrid origins (Table 1; Wagner et al., 1990). The unique characteristics of this taxon in combination with the fact that it is fully fertile suggest that it should be considered a distinct species. Although endemic to Kaua'i and O'ahu, the only known extant populations of this endangered taxon are found on Kaua'i

where they occur along waterfalls in the northeastern valleys that extend from the Alaka'i bog region and nearby Mt. Wai'ale'ale summit. Nuclear data place individuals of *P. princeps* var. *longibracteata* in the entirely *P. princeps* clade, while the chloroplast data firmly resolves them in the largely Kaua'i *P. pachyphylla* clade. These data, in combination with morphological, ecological, and reproductive characteristics, suggest that *P. princeps* var. *longibracteata* arose via homoploid hybrid speciation (Rieseberg, 1997). The putative parental types of this taxon are the woody shrub *P. princeps* var. *anomala* and an herbaceous *P. pachyphylla* form, both of which occur in the Alaka'i area. Gene flow between these taxa may have been considerable in the past given the close proximity of woodland and bog habitats in this area and these species' breeding and

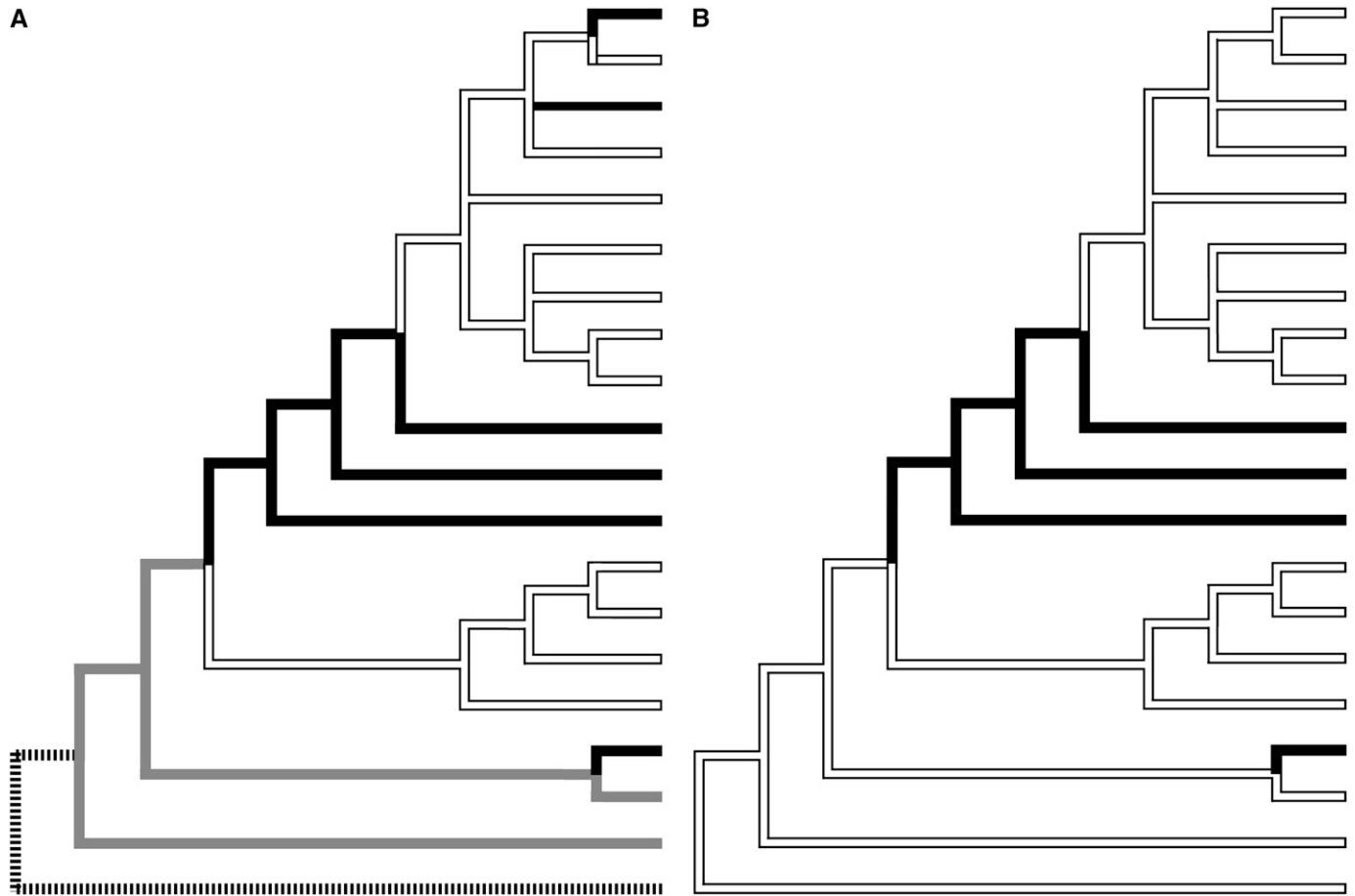


Fig. 4. Reconstruction of ancestral states mapped onto Bayesian consensus tree of the combined data set for Hawaiian *Plantago*. (A) Habitat (white = open bogs; black = woodlands; gray = coastal; dashed = unknown). (B) Growth form (white = herbaceous; black = woody).

pollination systems. Additionally, these taxa may have frequently been in contact until recently, before their severe reduction by feral ungulates in the latter part of the 1900s (USFWS, 1999).

Unlike *P. princeps* var. *longibracteata*, the morphological and ecological characteristics of the two other taxa are products of extensive gene flow via introgressive hybridization (Rieseberg, 1997). Nuclear data places O'ahu plants of *P. pachyphylla* in the clade containing Maui Nui and Hawai'i *P. pachyphylla* and *P. hawaiiensis* individuals, though the chloroplast data strongly supports its inclusion in the Kaua'i *P. pachyphylla* clade. We suggest that this result is due to initial derivation of this morphotype from an ancestral Kaua'i *P. pachyphylla* type followed by more recent gene flow with morphotypes of *P. pachyphylla* from the younger islands of Moloka'i and Maui.

The phylogenetic pattern exhibited by individuals of *P. princeps* var. *laxifolia* from 'Iao (West Maui) and Kipahulu (East Maui) similarly suggests contemporary gene flow with nearby *P. pachyphylla* morphotypes. The nuclear data place the 'Iao and Kipahulu individuals in a clade containing the other *P. princeps* taxa while the chloroplast data has them grouped with neighboring Pu'u Kukui (West Maui) and Hanawi (East Maui) *P. pachyphylla* forms, respectively. The data suggest that *P. princeps* var. *laxifolia* evolved from *P. princeps* var. *princeps* following dispersal of this taxon from Ko'olau to Maui Nui. The occurrence of populations of *P. princeps* var. *laxifolia* in the valleys directly below the mountain summits where *P. pachy-*

phylla populations are found, in combination with the high interfertility and reproductive systems of these taxa, insinuate that gene flow between them may have been common in the past when *P. princeps* var. *laxifolia* was more widespread than today (USFWS, 1999).

Taxonomic issues in Hawaiian *Plantago*—Hawaiian *Plantago* encompasses a range of habitats, growth forms, vegetative structures, and reproductive features, and this diversity has led to a lack of consensus regarding the group's taxonomy (Gray, 1862; Hillebrand, 1888; Rock, 1920; Pilger, 1923, 1936, 1937; Skottsberg, 1944; Wagner et al., 1990). Data presented here suggest that the current treatment by Wagner et al. (1990), recognizing three endemic species and four varieties, underrepresents diversity in the group. We found strong evidence that *P. pachyphylla* is paraphyletic, with morphotypes from Kaua'i forming a clade separate from those found on the younger islands. Within these clades, there is evidence of genetic divergence among *P. pachyphylla* morphotypes (see chloroplast and combined analyses), which, when corroborated by morphological, ecological, and geographic data, may be great enough to warrant their taxonomic separation. Additional taxonomic issues concern the *P. princeps* complex, particularly classification of *P. princeps* var. *longibracteata*. As previously noted, this taxon's unique morphology, novel habitat, fertility, and probable hybrid origins likely warrant elevating it to the species

level, following Pilger's (1923) classification. Furthermore, we found evidence of genetic divergence among Wai'anae and Ko'olau populations of the endangered *P. princeps* var. *princeps*, despite the fact that they are morphologically indistinguishable. It is possible that these populations represent cryptic or incipient species, a finding that would have significant conservation implications. Taken together, the taxonomic inconsistencies brought to light by this work necessitated taxonomic revisions, which are presently underway and to be published separately.

Conclusions—The apparent monophyly of Hawaiian *Plantago* along with its morphological and ecological diversity suggest that it is yet another example of adaptive radiation in the Hawaiian flora. Common patterns in Hawaiian plant adaptive radiations such as progressive, oldest to youngest, island colonization, allopatric speciation subsequent to interisland dispersal events and intransigent habitat shifts, and woodiness derived in situ from herbaceous ancestors have now been extended to Hawaiian *Plantago*, previously a relatively unknown and understudied group of Hawaiian plants. A novel finding from these results is the suggestion that a reversion from woodiness back to herbaceousness may have taken place, and if further substantiated, this would be unprecedented among adaptive radiation lineages in Hawai'i. This work further suggests that hybridization has played an important role in the evolutionary history of this lineage as it has in many other lineages within the Hawaiian Islands (Carr and Kyhos, 1986; Koutnik, 1990; Wagner et al., 1990; Howarth and Baum, 2005). Lastly, analyses presented here have confirmed the morphological complexity and reinforced the need for taxonomic revision of Hawaiian *Plantago*, a finding that will likely be extended to many other Hawaiian plant groups as studies of this nature become more frequent.

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APPENDIX 1. List of taxa used in this study, voucher information, and GenBank accession numbers for the four regions studied. Missing data are indicated with a dash (—). The following abbreviations are used for herbaria: BISH = Bishop Museum, PTBG = National Tropical Botanical Garden, OSC = Oregon State University, NY = New York Botanical Garden, WELTU = Victoria University.

Taxon; *Voucher*, Collection location; GenBank accessions: ITS; ETS; *ndhF-rpl32*; *rpl32-trnL*.

Plantago hawaiiensis (A. Gray) Pilg.; *Dunbar 19 PTBG*, Kipuka Maunaiu, (Hawaii) HI, USA; EU602319; EU583394; EU580409; EU594444. *Plantago hawaiiensis* (A. Gray) Pilg.; *Dunbar 219 PTBG*, Kulani, (Hawaii) HI, USA; EU602326; EU583401; EU580416; EU594451. *Plantago macrocarpa* Cham. & Schltdl.; *Woodbridge 181046 OSC*, Lincoln, OR, USA; EU602337; EU583412; EU580432; —. *Plantago pachyphylla* A. Gray; *Dunbar 296 PTBG*, Wainiha, (Kauai) HI, USA; EU602333; EU583408; EU580423; EU594458. *Plantago pachyphylla* A. Gray; *Dunbar 304 PTBG*, North Bog, (Kauai) HI, USA; EU602340; EU583415; EU580429; EU594464. *Plantago pachyphylla* A. Gray; *Dunbar 341 PTBG*, Waialeale, (Kauai) HI, USA; EU602334; EU583409; EU580424; EU594459. *Plantago pachyphylla* A. Gray; *Dunbar 345 PTBG*; Sincok Bog, (Kauai) HI, USA; EU602335; EU583410; EU580425; EU594460. *Plantago pachyphylla* A. Gray; *Dunbar 234 PTBG*, Poamoho, (Oahu) HI, USA; EU602327; EU583402; EU580417; EU594452. *Plantago pachyphylla* A. Gray; *Wagner 4918 BISH*, Pepeopae Bog, (Molokai) HI, USA; EU602320; EU583395; EU580410; EU594445. *Plantago pachyphylla* A. Gray; *Dunbar 245 PTBG*, Pelekunu, (Molokai) HI, USA; EU602341; EU583416; EU580430; EU594465. *Plantago pachyphylla* A. Gray; *Dunbar 100 PTBG*, Puu Kukui, (Maui) HI, USA; EU602324; EU583399; EU580414; EU594449. *Plantago pachyphylla* A. Gray; *Dunbar 140 PTBG*, Puu Kukui, (Maui) HI, USA; EU602323; EU583398; EU580413; EU594448. *Plantago pachyphylla* A. Gray; *Dunbar 274 PTBG*, Eke, (Maui) HI, USA; EU602330; EU583405; EU580420; EU594455.

Plantago pachyphylla A. Gray; *Dunbar 60 PTBG*, Hanawi, (Maui) HI, USA; EU602321; EU583396; EU580411; EU594446. *Plantago pachyphylla* A. Gray; *Henrickson 3598 BISH*, Geologist Bog, (Maui) HI, USA; EU602336; EU583411; EU580426; EU594461. *Plantago princeps* Cham. & Schltdl. var. *anomala* Rock; *Lorence 5134 PTBG*, Kalalau, (Kauai) HI, USA; EU602325; EU583400; EU580415; EU594450. *Plantago princeps* Cham. & Schltdl. var. *laxifolia* A. Gray; *Dunbar 111 PTBG*, Iao, (Maui) HI, USA; EU602322; EU583397; EU580412; EU594447. *Plantago princeps* Cham. & Schltdl. var. *laxifolia* A. Gray; *Wood 3214 PTBG*, Kipahulu, (Maui) HI, USA; EU602328; EU583403; EU580418; EU594453. *Plantago princeps* Cham. & Schltdl. var. *longibracteata* H. Mann; *Dunbar 289 PTBG* and *Perlman 16,425 PTBG*, Blue Hole, (Kauai) HI, USA; EU602331; EU583406; EU580421; EU594456. *Plantago princeps* Cham. & Schltdl. var. *longibracteata* H. Mann; *Wood 10,654 PTBG*, Iliiliula, (Kauai) HI, USA; EU602332; EU583407; EU580422; EU594457. *Plantago princeps* Cham. & Schltdl. var. *princeps*; *Perlman 17,181 PTBG*, Waiawa, (Oahu) HI, USA; EU602329; EU583404; EU580419; EU594454. *Plantago princeps* Cham. & Schltdl. var. *princeps*; *Perlman 15,594 PTBG*, Ekahanui, (Oahu) HI, USA; EU602342; EU583417; EU580431; EU594466. *Plantago rapensis* Pilg.; *Wood 9332 PTBG*, Rapa Island, French Polynesia; EU602339; EU583414; EU580428; EU594463. *Plantago rupicola* Pilg.; *Motley 2678 NY*, Rapa Island, French Polynesia; EU602338; EU583413; EU580427; EU594462. *Plantago spathulata* Hook.f.; *Garnock-Jones 2557 WELTU*, Wellington, New Zealand; EU602343; EU583418; EU580433; EU594467.