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Molecular phylogenetics and evolution of the endemic Hawaiian genus *Adenophorus* (Grammitidaceae)

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Abstract

Recent studies of the phylogeny of several groups of native Hawaiian vascular plants have led to significant insights into the origin and evolution of important elements of the Hawaiian flora. No groups of Hawaiian pteridophytes have been subjected previously to rigorous phylogenetic analysis. We conducted a molecular phylogenetic analysis of the endemic Hawaiian fern genus *Adenophorus* employing DNA sequence variation from three cpDNA fragments: *rbcL*, *atpβ*, and the *trnL-trnF* intergenic spacer (IGS). In the phylogenetic analyses we employed maximum parsimony and Bayesian inference. Bayesian phylogenetic inference often provided stronger support for hypothetical relationships than did nonparametric bootstrap analyses. Although phylogenetic analyses of individual DNA fragments resulted in different patterns of relationships among species and varying levels of support for various clades, a combined analysis of all three sets of sequences produced one, strongly supported phylogenetic hypothesis. The primary features of that hypothesis are: (1) *Adenophorus* is monophyletic; (2) subgenus *Oligadenus* is paraphyletic; (3) the enigmatic endemic Hawaiian species *Grammitis tenella* is strongly supported as the sister taxon to *Adenophorus*; (4) highly divided leaf blades are evolutionarily derived in the group and simple leaves are ancestral; and, (5) the biogeographical origin of the common ancestor of the *Adenophorus–G. tenella* clade remains unresolved, although a neotropical origin seems most likely.
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1. Introduction

The vascular plant flora of the Hawaiian Islands is characterized by having one of the highest levels of endemism of any geographic region in the world. Approximately 89% of angiosperm species and 76% of pteridophyte species are endemic to the archipelago (Ranker et al., 2000, and references therein; Wagner et al., 1990). The lure of endemism combined with spectacular phylogenetic radiations of species has led to some of our most insightful studies of plant evolution and biogeography (e.g., see Wagner and Funk, 1995, and references therein). For many groups of angiosperms in Hawaii, we now have strong evidence for members of species-rich and morphologically/ecologically diverse groups being descendants of single colonizing ancestors. For example, evidence from multiple kinds of data suggests that the 28 endemic species of the Hawaiian silversword alliance (Asteraceae) arose from a single ancestor (e.g., Baldwin et al., 1991). Other well-studied examples include the Hawaiian lobelioids (Campanulaceae) with 98 species (Givnish et al., 1995), Hawaiian *Pittosporum* (Pittosporaceae) with 11 species (Gemmill et al., 2002), *Tetramolopium* (Asteraceae) with 11 Hawaiian species (Lowrey, 1995), and *Schiedea* and *Alsinidendron* (Caryophyllaceae) that comprise an endemic group of 28 species (Wagner et al., 1995b). Numerous other groups of Hawaiian flowering plants also appear to have evolved from single colonizing ancestors (Fosberg, 1948; Wagner et al., 1990).

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Generic endemism of Hawaiian angiosperms is 15% (32/216), the highest in the world. By contrast, only three (5.6%) of the 54 genera of native Hawaiian pteridophytes are considered endemic (D. Palmer, pers. comm.), and the members of each are presumed to have evolved from single colonizing ancestors (Bishop, 1974; Fosberg, 1948; Wagner, 1981). Most species of Hawaiian pteridophytes are classified in widespread genera, where the number of colonizing ancestors is simply not evident. Even though pteridophytes represent about 16% of the native species of vascular plants in Hawaii (Fosberg, 1948; Wagner, 1988), they have been relatively poorly studied, particularly in terms of phylogenetic systematics. Following the seminal work of Wagner (1952) on the endemic genus *Diellia* (Aspleniaceae), wherein he produced one of the first formal phylogenetic analyses of any group of organisms, no further phylogenetic studies of Hawaiian pteridophytes have been published. Although Wagner (1952) attempted to resolve species relationships within *Diellia*, he did not conduct an outgroup analysis in an attempt to discover the closest living non-Hawaiian relatives of the genus. Herein we describe our study of one of the other three putatively endemic genera of pteridophytes in Hawaii, the genus *Adenophorus* Gaudich. (Grammitidaceae). This study represents the first broadly based phylogenetic analysis of any group of Hawaiian pteridophytes.

Grammitidaceae Newman is a tropical/subtropical family of primarily epiphytic ferns. The family comprises approximately 750 species and is characterized by green tetrahedral spores, sporangial stalks usually of only one row of cells, and leaf traces of single vascular strands (Parris, 1990, 1998b). This group of species has been recognized at a variety of taxonomic levels, however, the circumscription and monophyly of the group has seldom been questioned. By contrast, there has been little agreement among workers in this group on generic circumscriptions. For example, Parris (1990) recognized only four genera in the family, whereas, Copeland (1947) recognized 12, and Parris (1998b) currently recognizes over 20 genera. Smith (1993) recognized 10 genera of Grammitidaceae for the New World. The present study is part of a larger effort to define monophyletic groups of species in Grammitidaceae using phylogenetic analyses of a combination of molecular and morphological characters.

The Hawaiian genus *Adenophorus* has been recognized as distinct from other members of Grammitidaceae based primarily on the presence of putatively unique glandular, receptacular paraphyses (Bishop, 1974). [Receptacular paraphyses are defined as sterile organs arising from the receptacle (Parris, 1997).] The paraphyses of *Adenophorus* spp. typically comprise a uniseriate column of 2-several cells with a much enlarged glandular, apical cell. Parris (1997, 1998a), however, noted the similarity of the paraphyses of *Adenophorus* species to those of *Chrysogrammitis* Parris from southeast Asia/Melanesia, *Grammitis rigida* Hombr. of New Zealand, or possibly to those of various species of *Ctenopteris* (see Baayen and Hennipman, 1987). In addition, glandular receptacular paraphyses have been observed in the Hawaiian endemic *Grammitis tenella* Kaulf., although the apical cell is typically smaller than those observed in *Adenophorus* species (Parris, 1997; Wagner, 1964); however, it has never been suggested that *G. tenella* is closely related to *Adenophorus*. In fact, the possible phylogenetic relationships of *G. tenella* have not been critically assessed by any investigator. Although the homology of the glandular paraphyses across the taxa mentioned above is unknown, their taxonomic distribution could be of phylogenetic significance in indicating a close relationship of *Adenophorus* species to at least some species presently treated in other grammitid genera.

Bishop (1974) classified the species of *Adenophorus* into two morphologically distinct subgenera: subg. *Adenophorus*, with four species characterized by 2- to 3-pinnatifid or pinnate-pinnatifid leaves and the absence of root buds (*A. abietinus* (D.C. Eaton) K.A. Wilson, *A. hymenophylloides* (Kaulf.) Hook. and Grev., *A. tamariscinus* (Kaulf.) Hook. and Grev., and *A. tripinnatifidus* Gaudich.) and subg. *Oligadenus* L.E. Bishop, with four species characterized by simple to pinnatifid leaves and the presence of adventitious root buds (*A. haalilioanus* (Brack.) K.A. Wilson, *A. oahuensis* (Copel.) L.E. Bishop, *A. periens* L.E. Bishop, and *A. pinnatifidus* Gaudich.; however, the presence of root buds had not been verified in *A. periens*). Bishop (1974) recognized two distinctive varieties, in addition to the type variety, within *A. tamariscinus*: var. *epigaeus* and var. *montanus*. Wagner et al. (1995b) elevated those varieties to species rank in the belief that populations of those taxa were consistently and sufficiently morphologically distinct to warrant such a classification.

The goals of the present work were to: (1) assess the monophyly of *Adenophorus* and of the two subgenera of Bishop; (2) estimate phylogenetic relationships within *Adenophorus*; (3) determine the closest living relatives of *Adenophorus* and, thus, investigate the biogeographical origins of the group; (4) explore molecular and morphological evolution of the species within a phylogenetic context; and, (5) assess relationships of the controversial and peculiar *Grammitis tenella*.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Outgroup relationships of *Adenophorus* were estimated in a larger maximum parsimony, phylogenetic

analysis of 51 grammitid sequences of the chloroplast (cp) DNA gene *rbcL* and of the cpDNA *atpβ* gene, with one non-grammitid outgroup, *Pteridium aquilinum* (L.) Kuhn; sequences for the outgroup were available from GenBank. The details and results of that larger analysis will be presented elsewhere. Of the taxa surveyed, a strongly supported monophyletic group, comprising *Grammitis bryophila* (Maxon) F. Seym., *G. melanoloma* (Cordem.) Tardieu, *Cochlidium punctatum* (Raddi) L.E. Bishop, and *C. rostratum* Maxon ex C. Chr., was supported as the closest non-Hawaiian relative of *Adenophorus* with 95% bootstrap support. We found that we could employ any one or any combination of those taxa to resolve relationships within *Adenophorus* and always achieved the same result; thus, to simplify analyses, we used only sequences from *G. bryophila* as an outgroup to resolve relationships within *Adenophorus*. To assess the level of support for the sister-taxon status of the *G. bryophila* clade to *Adenophorus*, we employed sequences of *Terpsichore eggersii* (Baker ex Hook.) A.R. Sm. as a further outgroup. This had been shown in our broader analyses to belong to another clade sister to the *Adenophorus–Cochlidium–Grammitis* subg. *Grammitis* clade/alliance. Samples of all species of *Adenophorus* were collected fresh in Hawaii, along with the Hawaiian endemic *Grammitis tenella*. Leaf material was stored in silica gel until DNA was extracted. Samples of non-Hawaiian taxa were supplied by several colleagues (Table 1). Table 1 lists locality, collector, collection number, herbarium of deposit for voucher specimens, and GenBank accession numbers for all taxa and DNA sequences studied, including outgroup species. We extracted total cellular DNA using the CTAB method of Doyle and Doyle (1987) modified by adding 3% PVP-40 and 5 mM ascorbic acid. Sample DNA concentrations were standardized to 10 mg/mL with the aid of a mini-fluorometer.

2.2. PCR amplification and sequencing

We PCR-amplified and sequenced three segments of the cpDNA genome: a 1311 basepair (bp) fragment of the *rbcL* gene, a 1266 bp fragment of the *atpβ* gene, and the *trnL-F* intergenic spacer (IGS). PCR amplification of *rbcL* was accomplished with a combination of primers synthesized from sequences given in Zurawski et al. (1981) and Wolf et al. (1994), and those designed specifically for this study (Table 2). We amplified *rbcL* as three, overlapping fragments with the primer pairs Z1 + G673R, F673F + Z1351R, and G454F + G1204R; sequencing primers were G10F, G683F, and G1195R, respectively. PCR conditions were described in Haufler and Ranker (1995). We used PCR primer sequences for *atpβ* amplifications from Wolf (1997) or modified therefrom (Table 2). We amplified four overlapping fragments with various combinations of primer pairs, as follows with sequencing primers in brackets: (1) 470F + 609R [470F], (2) 672F2 + 1334R [672F2, 888F, or 965F], or 905F + 1334R [905F], (3) 1163F2 + 1592R [1163F2], and (4) 1419F2 + 1365R [1419F2]. PCR conditions were 94 °C (180 s), followed by 30–35 cycles of 94 °C (90 s), 42 °C (120 s), and 72 °C (180 s), ending with 10 min at 72 °C after cycling was completed. Amplification of the *trnL-F* IGS was achieved with primers "e" and "f" of Taberlet et al. (1991). PCR conditions were 94 °C (180 s), followed by 5 cycles of 94 °C (30 s), 45 °C (30 s), and 72 °C (30 s), followed by 37 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s), ending with 10 min at 72 °C after cycling was completed. The same primers were used individually for sequencing each strand of the spacer. PCR products were purified with the Promega Wizard PCR Preps Purification System. Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Kit, employing 1/4 reactions. Sequencing products were purified with G-50

Table 1
Species list, collection and voucher information, and GenBank accession numbers

Species	Locality, collector, number, and herbarium	GenBank accession numbers		
		rbcL	*atpβ*	*trnL-F* spacer
Adenophorus abietinus	USA, Hawaii, Kauai, Ranker 1100, COLO	AF468202	AF469778	AF469791
Adenophorus epigaeus	USA, Hawaii, Kauai, Ranker 1103, COLO	AF468203	AF469779	AF469792
Adenophorus haalilioanus	USA, Hawaii, Oahu, Ranker 1561, COLO	AF468200	AF469775	AF469788
Adenophorus hymenophylloides	USA, Hawaii, Hawaii, Ranker 1203, COLO	AF468204	AF469780	AF469793
Adenophorus montanus	USA, Hawaii, Molokai, Ranker 1351, COLO	AF468205	AF469781	AF469794
Adenophorus oahuensis	USA, Hawaii, Oahu, Ranker 969, COLO	AY057382	AF469776	AF469789
Adenophorus periens	USA, Hawaii, Hawaii, Ranker 1114, COLO	AF468199	AF469774	AF469787
Adenophorus pinnatifidus	USA, Hawaii, Oahu, Ranker 1559, COLO	AF468201	AF469777	AF469790
Adenophorus tamariscinus	USA, Hawaii, Hawaii, Ranker 1031, COLO	AF468206	AF469782	AF469795
Adenophorus tripinnatifidus	USA, Hawaii, Kauai, Ranker 1102, COLO	AF468207	AF469783	AF469796
Grammitis tenella	USA, Hawaii, Molokai, Ranker 1352, COLO	AF468198	AF469773	AF469786
Grammitis bryophila	Costa Rica, A. Rojas et al. 3240, UC	AF468208	AF469784	AF469797
Terpsichore eggersii	Dominica, S. R. Hill 29109, UC	AF468209	AF469785	AF469798

Table 2
Primer sequences used for PCR amplification and sequencing of *rbcL* and *atpβ*

rbcL	
Z1:	ATG TCA CCA CAA ACA GAA ACT AAA GCA AGT
G10F:	CAA ACA GAA ACT AAA GCA AGT GTT GGA TTC
G673F:	CTT TTC AAA TCC CAA GCT GAA
G673R:	TTC AGC TTG GGA TTT GAA AAG
G683F:	CCC AAG CTG AAA C(A/G)G GGG AAA TC
G454F:	CC(T/C) CAT GGT ATT CAG GTT GAA
G1204R:	CAA GGA TG(G/A) CCT AAG GTT CCT CCG CCG AA
G1195R:	GGT TCC TCC GCC GAA CTG TAA TAC
Z1351R:	CTT CAC AAG CAG CAG CTA GTT CAG GAC TCC
atpβ	
470F (73):	CAA ATC ATY GGW CCR GTA YTG GAT G
672F2 (275):	TTG ATA CGG GAG CCC CTC TCA GTG T
609R (632):	TCR TTD CCT TCR CGT GTA CGT TC
1163F2 (766):	ATG GCT GAA TAY TTY CGA GAT GTT A
1334R (937):	TTC TTT CYT GYA AAG AWC CCA TTT C
1419F2 (1022):	CRA CAT YTG CAC AYT TAG AYG CNA C
1592R (1195):	TGT AAC GYT GYA AAG TTT GCT TAA C
1365R (1387):	RAA GRA TCA TTT GAA ATC CCT T

Sequences are 5′–3′. Primers beginning with "G" were specifically designed for this study. Many were modified from sequences given in Wolf et al. (1994) and Wolf (1997). Numbering system for *atpβ* primers follows Wolf (1997); inferred position in gene of first 5′ position of primer is given parenthetically.

Sephadex columns from Amersham Pharmacia Biotech. Sequences were detected on ABI automated sequencers at the Iowa State University DNA Sequencing and Synthesis Facility. GenBank accession numbers for all sequences are in Table 1.

2.3. Phylogenetic analyses

Sequence fragments were edited by visual inspection of electropherograms in Sequencher (Gene Codes) and aligned manually (*rbcL* and *atpβ*) or with ClustalX (Thompson et al., 1997) and then manually adjusted to achieve more parsimonious alignments (*trnL-F* IGS).

We first conducted maximum parsimony phylogenetic analyses as implemented in PAUP* 4.0b8 (Swofford, 1998) for each set of DNA sequences separately. All characters were unordered and equally weighted. We found that weighting based on the phylogenetic information content of transitions vs transversions or of different codon positions produced the same tree topologies as equally weighted characters (results not shown). We employed the branch-and-bound algorithm with Mul-Trees activated. We performed nonparametric bootstrap analyses with the branch-and-bound algorithm with 5000 replications each. For analysis of the *trnL-F* IGS, sites in which gaps were inferred were excluded. We performed decay analyses (Bremer, 1988; Donoghue et al., 1992) with AutoDecay 4.0.1 (Eriksson, 1998).

Clade credibility values were estimated for each single data set and the combined data set calculating the posterior probability (PP) for each node using Bayesian inference with a Markov-chain Monte Carlo sampling method as implemented in MrBayes 2.1 (Huelsenbeck and Bollback, 2001; Huelsenbeck and Ronquist, 2001). One out of every 100 trees was sampled for 500,000 generations with kappa and DNA substitution parameters estimated during the search. The consensus tree was computed in PAUP* on the last 4000 sampled trees, excluding the 1000 trees found in the "burn-in period". PP values above $p = 0.95$ are considered to be statistically significant (Huelsenbeck and Ronquist, 2001; Larget and Simon, 1999; Lewis, 2001; Rannala and Yang, 1996). In each analysis, we ran four simultaneous MCMC chains.

2.4. Test of data partition incongruence

We employed the incongruence length difference test (ILD; Farris et al., 1994, 1995) as implemented in PAUP* (partition homogeneity test) to test the null hypothesis that our three data sets were homogeneous with respect to phylogenetic information. Invariant sites were removed for the test (Cunningham, 1997) and 10,000 replications were performed.

2.5. Estimation of nucleotide substitution patterns

Because the ILD test was not significant (Section 3), indicating that we could not reject the null hypothesis of data set homogeneity of phylogenetic information, we estimated the nucleotide substitution patterns using the combined data set via the maximum-likelihood algorithm as implemented in PAUP*. Only ingroup taxa were included in these analyses. MODELTEST version 3.06 (Posada and Crandall, 1998) was employed to find the model of DNA substitution that best fitted the data.

Hierarchical likelihood ratio tests suggested that the HKY model (Hasegawa et al., 1985) was most likely, whereas the Akaike information criterion method suggested that the general time-reversible model (Rodríguez et al., 1990; Yang, 1994) was most likely. We employed both models to estimate nucleotide substitution patterns among ingroup taxa.

3. Results

All analyses strongly supported a sister-taxon relationship between *Adenophorus* spp. and the Hawaiian endemic *Grammitis tenella*; thus, in subsequent discussion, we will consider the "ingroup" to include *G. tenella*.

3.1. Sequence variation

Of the 1311 bp of the *rbcL* gene sequenced, 70 sites were variable among ingroup species and 24 sites were phylogenetically informative; the sequences we analyzed began at position 40 of the gene, relative to *Zea mays* (Zurawski et al., 1981) (Table 3). Of the 1266 bp of the *atpβ* gene, 54 sites were variable and 19 sites were phylogenetically informative among ingroup species; the sequences began at position 100 of the gene, relative to rice and tobacco (see Wolf, 1997). All *rbcL* and *atpβ* sequences were aligned without inferring insertions or deletions (indels). Most phylogenetically informative mutations were silent (i.e., synonymous) with respect to amino acid substitutions, however, nonsynonymous substitutions were inferred at three positions for *rbcL* and four positions for *atpβ* (see Section 4). Lengths of the *trnL-F* IGS varied among ingroup species from 278 to 283 bp and were 278 and 297 bp in the outgroups *G. bryophila* and *Terpsichore eggersii*, respectively. Total length of aligned IGS sequences was 303 bp due to the insertion of gaps to achieve an alignment of maximum similarity among taxa. Thus, of the 264 aligned sites (that is, excluding 39 gapped sites), 38 were variable across ingroup species and 14 were phylogenetically informative. The percentage of variable sites of each total fragment length across ingroup

taxa was nearly identical for *rbcL* and *atpβ* (5.3 and 4.3%, respectively) but was considerably higher for *trnL-F* IGS (14.2%). By contrast, the percentage of informative sites out of the number of variable sites for each set of DNA fragments was nearly identical (34–37%; Table 3). The same patterns were exhibited in each set of fragments when outgroups were included (Table 3).

Uncorrected sequence divergences between species of *Adenophorus* ranged from 0.5 to 2.5% (mean 1.3%) for *rbcL*, 0.0 to 1.7% (mean 1.0%) for *atpβ*, and 0.0 to 7.8% (mean 3.8%) for *trnL-F* IGS (the last were estimated omitting sites with gaps). Sequence divergences between *G. tenella* and *Adenophorus* spp. ranged from 1.4 to 2.4% for *rbcL*, 1.3 to 1.8% for *atpβ*, and 2.2 to 6.2% for *trnL-F* IGS. Divergences between the outgroup *G. bryophila* and ingroup species ranged from 2.8 to 4.0% for *rbcL*, 2.9 to 3.7% for *atpβ*, and 11.3–16.6% for *trnL-F* IGS. Divergences between the far outgroup *T. eggersii* and all other taxa ranged from 3.7 to 4.8% for *rbcL*, 4.3 to 5.1% for *atpβ*, and 10.6 to 17.6% for *trnL-F* IGS.

3.2. Phylogenetic relationships

Although our phylogenetic analyses are based on cpDNA sequences only, we will discuss putative relationships among species.

3.2.1. rbcL analysis

Parsimony analysis of *rbcL* sequences produced one most parsimonious tree ($L = 160$, CI $= 0.81$, RI $= 0.58$; Fig. 1), in which all relationships were dichotomously resolved. An identical consensus tree resulted from the Bayesian analysis (Fig. 1). *Grammitis tenella* was supported as sister to *Adenophorus*, but with only 56% bootstrap support and a Bayesian PP of 76 ($= 0.76$). Within *Adenophorus*, subg. *Adenophorus* of Baldwin et al. (1991) was supported as a monophyletic group, with only 53% bootstrap support but with a PP of 92. Three species in subg. *Oligadenus* (*A. oahuensis*, *A. haalilioanus*, and *A. pinnatifidus*) were strongly supported as a monophyletic clade (here called the 'Oligadenus clade;' 90% bootstrap, PP $= 100$), sister to subg. *Adenophorus*. The fourth species of subg. *Oligadenus*, *A.*

Table 3
Sequence variation by DNA fragment

Fragment	Total length sequenced	Ingroup only		All taxa	
		No. of variable sites (% of total)	No. of informative sites (% of var. sites)	No. of variable sites (% of total)	No. of informative sites (% of var. sites)
rbcL	1311	70 (5.3%)	24 (34.3%)	85 (6.5%)	36 (42.4%)
atpβ	1266	54 (4.3%)	19 (35.2%)	80 (6.3%)	35 (43.8%)
trnL-F IGS	278–283	38 (14.2%)	14 (36.8%)	51 (19.3%)	19 (37.3%)

Note. For *trnL-F* IGS, length of sequences as aligned with outgroups was 303 bp. Percentages were calculated after the omission of 39 sites in which gaps were inserted in some sequences (264 bp in analysis).
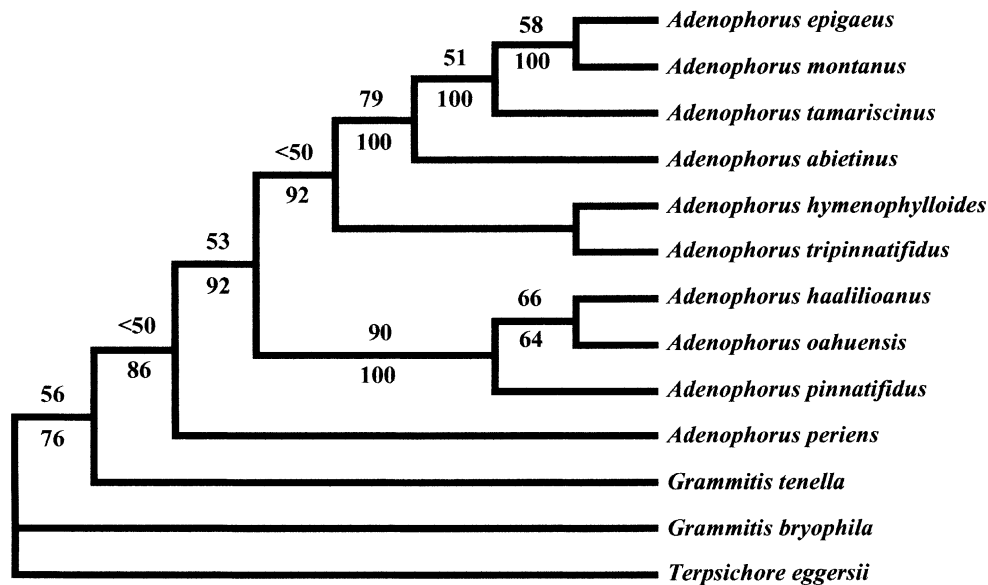
Fig. 1. Single most-parsimonious tree resulting from the branch-and-bound maximum parsimony analysis of the *rbcL* dataset. The topology is identical to the consensus tree resulting from the Bayesian analysis. Numbers above branches are percent bootstrap support. Numbers below branches are Bayesian posterior probabilities (×100).

periens, was weakly supported as being sister to a clade consisting of all other species of *Adenophorus* (bootstrap <50%; PP = 86). Within subg. *Adenophorus*, bootstrap support for relationships was generally <60% except for that supporting the monophyly of the three species treated by Bishop (1974) as subspecies of *A. tamariscinus* (*A. tamariscinus*, *A. epigaeus*, and *A. montanus*; here called the '*A. tamariscinus* clade') plus *A. abietinus*, with 79% bootstrap support (Fig. 1). By contrast, posterior probabilities from Bayesian inference were generally much higher, with three branches that have PP = 100 (Fig. 1).

3.2.2. atpβ analysis

Parsimony analysis of *atpβ* sequences produced four equally parsimonious trees (L = 131, CI = 0.90, RI = 0.78), a strict consensus of which is shown in Fig. 2A. Relationships that were supported in common with the *rbcL* analysis, and for which there was high bootstrap support, include the sister-taxon relationship between *G. tenella* and *Adenophorus* (97% bootstrap), the monophyly of *Adenophorus* (88% bootstrap), the monophyly of the *A. tamariscinus* clade (80% bootstrap), the sister-taxon relationship between the latter clade and *A. abietinus* (80%), monophyly of the *Oligadenus* clade (100%), and a sister-taxon status of *A. oahuensis* and *A. haalilioanus* (99%). In fact, the latter two species were identical for the *atpβ* sequence, although their *rbcL* sequences differed by 0.7% (Table 3). The relationships of *A. hymenophylloides*, *A. periens*, and *A. tripinnatifidus* were not resolved in the strict consensus tree based on analysis of *atpβ* sequences.

Bayesian analysis of the *atpβ* data set suggested similar relationships as was found through parsimony analysis, with a few important differences (Fig. 2B). The primary differences involved the placement of the species *A. periens*, *A. tripinnatifidus*, and *A. hymenophylloides*.

3.2.3. trnL-F IGS analysis

Parsimony analysis of the IGS produced the least resolved of the three single-sequence analyses. Four equally parsimonious trees were found (L = 87, CI = 0.91, RI = 0.81), a strict consensus of which is shown in Fig. 3. Identical IGS sequences were discovered for *A. abietinus*, *A. epigaeus*, *A. montanus*, and *A. tamariscinus*. Similarly, identical sequences were shared by *A. haalilioanus* and *A. oahuensis*. The phylogenetic analysis of this region provided strong bootstrap support only for the monophyly of those two groups of species. The sequence shared by *A. haalilioanus* and *A. oahuensis* was distinguished from other IGS sequences by a 6 bp insertion (positions 42–47, as aligned and polarized by outgroup comparison) and a 1 bp deletion (position 124). [NB: Indels were not included in the analysis but simply mapped onto the consensus tree.] The monophyly of the clade *Grammitis tenella* plus *Adenophorus* was supported by a bootstrap value of 60% and a 1 bp deletion at position 122. Although the monophyly of *Adenophorus* was poorly supported by phylogenetic analysis of IGS sequences, all species of the genus shared a 3 bp insertion at positions 48–50. Support for the resolved branches via Bayesian inference was generally greater than or equal to that from parsimony bootstrap (Fig. 3).
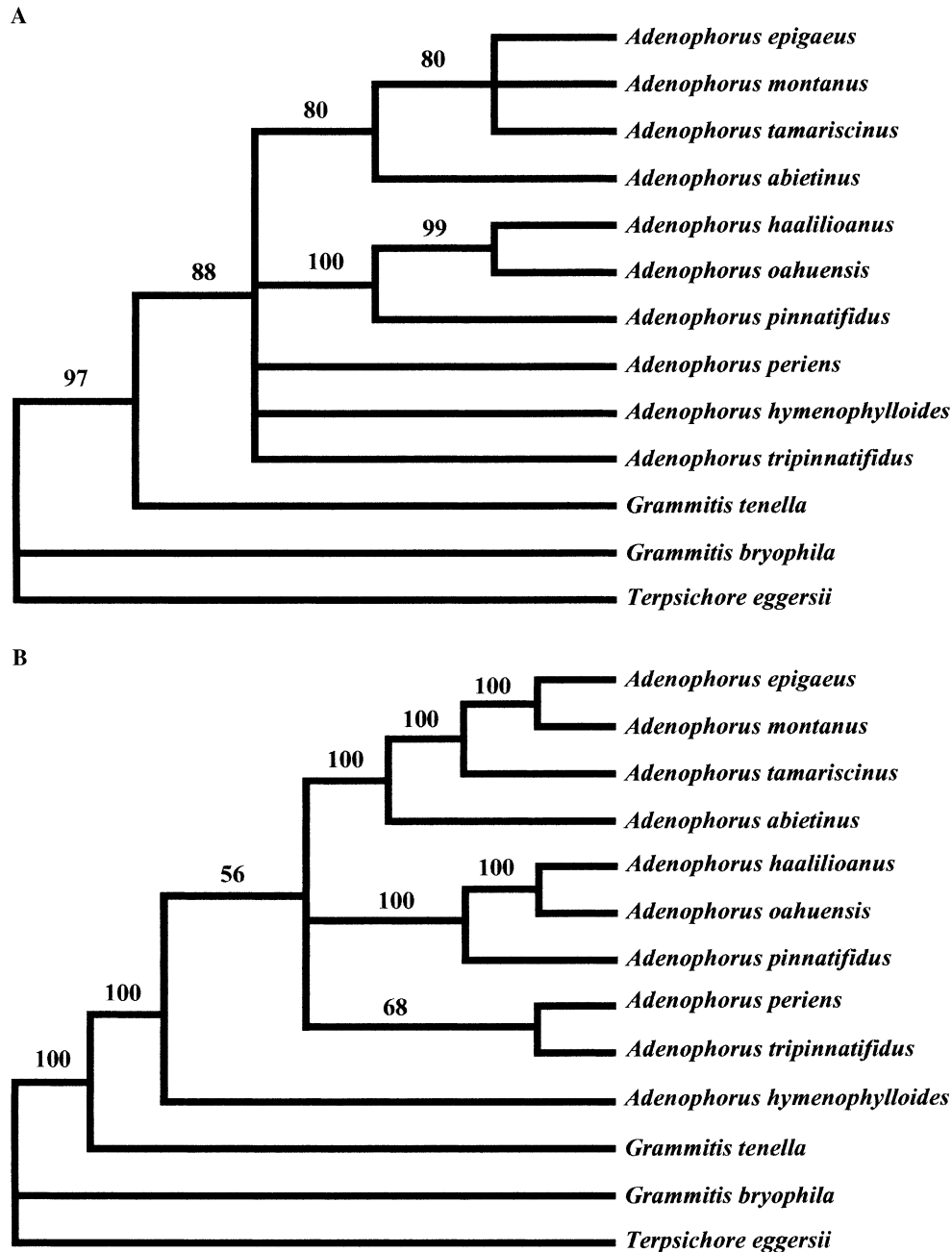
A

B

Fig. 2. (A) Strict consensus tree of the four equally parsimonious trees resulting from branch-and-bound maximum parsimony analysis of the *atpβ* dataset. Numbers above branches are percent bootstrap support. (B) Consensus tree resulting from Bayesian analysis of the *atpβ* dataset. Numbers above branches are Bayesian posterior probabilities ($\times 100$).

3.2.4. Data incongruence

The result of the ILD test was not significant ($p = 0.87$), indicating that we could not reject the null hypothesis of data set homogeneity. Based on this result, we combined all three chloroplast DNA sequences into one data set and performed a parsimony analysis as described above.

3.2.5. Combined analysis

Parsimony analysis of the combined data set resulted in one most parsimonious tree ($L = 379$, CI $= 0.86$,

RI $= 0.70$; Fig. 4) in which all relationships were dichotomously resolved. The topology was the same as that of the *rbcL* tree, although the branches generally had much higher support. Even though most branches were strongly supported by both bootstrap values and decay indices (DI), several were relatively weakly supported. Only one branch was supported by a PP value of less than 98 via Bayesian inference (Fig. 4). The potential sister-taxon relationship between the pair *A. hymenophylloides* and *A. tripinnatifidus* and the remainder of subg. *Adenophorus* was supported only by a 54%
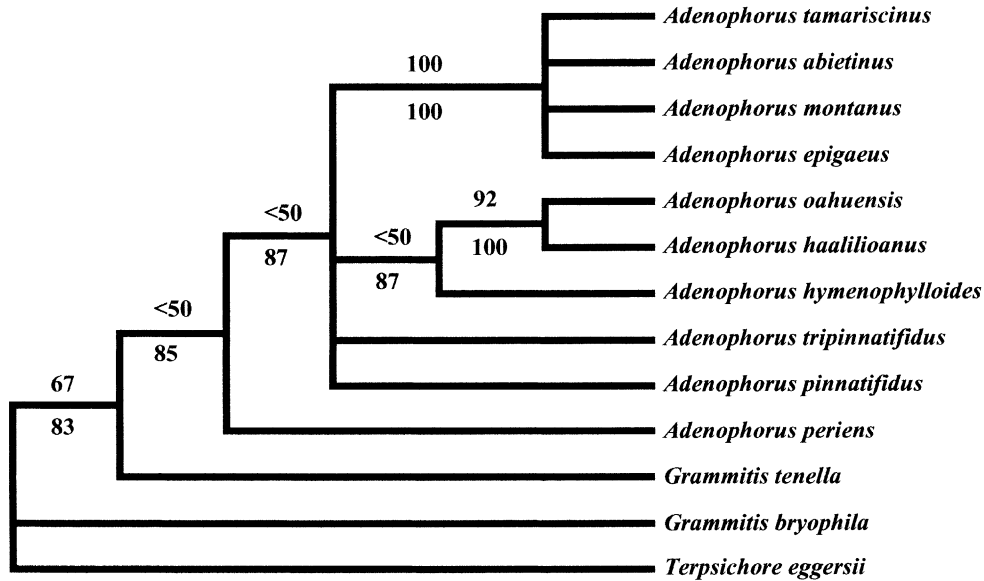
Fig. 3. Strict consensus tree of the four equally parsimonious trees resulting from branch-and-bound maximum parsimony analysis of the *trnL-F* IGS dataset. Numbers above branches are percent bootstrap support. Numbers below branches are Baysian posterior probabilities (×100).
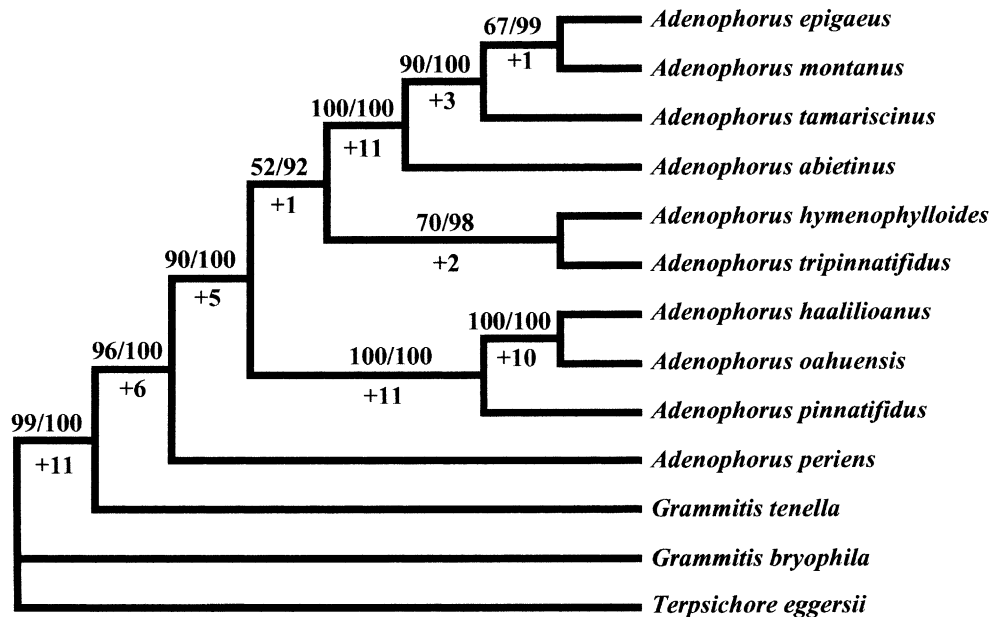

Fig. 4. Single most-parsimonious tree resulting from the branch-and-bound maximum parsimony analysis of the 3-sequence, combined dataset. The topology is identical to the consensus tree resulting from the Baysian analysis. Numbers above branches are percent bootstrap support/Baysian posterior probabilities (×100). Numbers below branches are decay indices.

bootstrap value, DI of one, and a PP of 92. Similarly, a DI of one and a bootstrap value of only 67% supported the hypothetical sister-taxon status of *A. epigaeus* and *A. montanus*; that relationship was supported, however, by a PP of 99. The next highest support was for the sister-taxon relationship of *A. hymenophylloides* and *A. tripinnatifidus*, with 70% bootstrap, a DI of two, and a PP of 98. All other branches were supported by at least 89% bootstrap values, DI ⩾ 3, and PP values of 100 (Fig. 4).

4. Discussion

4.1. Monophyly of Adenophorus and of subgenera Adenophorus and Oligadenus

Our phylogenetic analyses of molecular data strongly support the monophyly of *Adenophorus*, thus suggesting that this is truly an endemic group to the Hawaiian Islands and that all extant species share a unique common

ancestor. Although phylogenetic analysis of the *trnL-F* IGS sequences alone provided only weak support for the monophyly of the genus, all *Adenophorus* species shared a 3 bp inferred insertion (positions 48–50 of the aligned sequences) relative to *G. tenella* and outgroup taxa. That information, in concert with the results of the phylogenetic analysis of the combined data set, provides robust support for the monophyly of the whole genus. The monophyly of each subgenus described by Bishop (1974), however, is less certain. Within subg. *Adenophorus*, the combined analysis provided 100% bootstrap and Bayesian support for the monophyly of a four-species clade, however, the phylogenetic relationships of the pair *A. hymenophylloides–A. tripinnatifidus* were less well supported and their relationships remain uncertain based on molecular data. Subgenus *Oligadenus* is clearly paraphyletic based on molecular data with the single species *A. periens* being supported as sister to all other species of *Adenophorus* and the remaining three species comprising a distinct and well-supported clade. In summary, our data and analyses do not provide strong support for the subgeneric classification of Bishop (1974).

4.2. Phylogenetic relationships and evolution within Adenophorus

The low levels of molecular divergence and, thus, the inferred close relationships among the three species of the *A. tamariscinus* clade, *A. epigaeus*, *A. montanus*, and *A. tamariscinus*, are perhaps not surprising in light of their high morphological similarity to each other as reflected in the classification of Bishop (1974), wherein he considered these taxa as subspecies of *A. tamariscinus*. The pair-wise molecular distances among members of this clade were among the lowest of all species comparisons within the genus. Distances ranged from 0.5% to 1.0% for *rbcL* and from 0.1% to 0.2% for *atpβ*. The taxa shared identical sequences for the *trnL-F* IGS. Bishop also noted the extreme morphological similarity between *A. abietinus* and *A. tamariscinus* s. l., the former differing primarily from the latter in being smaller in all dimensions; this is even true when they are growing side-by-side on the same tree (personal observations). For DNA sequences, *A. abietinus* shared the same *trnL-F* IGS sequence with the three species of the *A. tamariscinus* clade and exhibited little divergence for *rbcL* (mean distance 0.6%) and *atpβ* (mean distance 0.5%). Morphologically, the four taxa share the unique features within the genus of having xylem leaf traces being double or branched (vs a single arc in all other ingroup species) and rhizome scales that are narrowly triangular (vs deltate, sublinear, or linear-lanceolate in other ingroup species). *Adenophorus epigaeus* is distinguished from the other species by having much longer rhizome internodes than other taxa in this clade, and *A. mont-*

anus typically is distinguished by having apical, marginal sori that extend onto both abaxial and adaxial leaf surfaces (vs strictly abaxial sori in the other taxa) (personal observations; Bishop, 1974). There were several inferred synapomorphic and autapomorphic mutations that resulted in apparent amino acid substitutions for *rbcL* and *atpβ* and that add support to the relationships and distinctness of the species in this group. The sister-species status of *A. epigaeus* and *A. montanus* is supported by a shared mutation at position 1214 of *rbcL*, which is in a 2nd-codon position, and that would result in a change from glutamic acid to lysine (a negatively to a positively charged residue). The sister-taxon status of *A. tamariscinus* with the two former species is supported by a 1st-codon-position mutation at position 202 of *rbcL* and that would result in a substitution of threonine with a serine residue (both polar). In addition, *A. epigaeus* had one unique, autapomorphic *rbcL* amino acid substitution, *A. tamariscinus* had five unique, autapomorphic *rbcL* amino acid substitutions, and *A. abietinus* had one such substitution in each of *rbcL* and *atpβ*. Although a more critical assessment of the specific distinction of the members of the *A. tamariscinus* clade + *A. abietinus* may be warranted, we suggest that the taxa may be sufficiently morphologically and molecularly distinct to each merit species status. Their high morphological and molecular similarity may be a reflection of relatively recent divergence and radiation from common ancestors.

Adenophorus oahuensis and *A. haalilioanus* are nearly morphologically identical, thus, we expected to find a close relationship between these two species. Both species have simple leaves 2–10 cm long that usually have entire, unlobed margins in the former and regularly lobed or crenate margins in the latter. Another common, but not fixed, difference is that in *A. oahuensis* paraphyses are often not gland-tipped or even absent entirely, whereas in *A. haalilioanus* paraphyses are always gland-tipped. This high degree of morphological similarity is consistent with the two taxa having identical DNA sequences for *atpβ* and *trnL-F* IGS. Their shared *atpβ* sequence has a unique 1st-codon-position mutation at position 146 that would produce a substitution of an isoleucine for a valine residue (both nonpolar) compared to other taxa. Also, their shared *trnL-F* IGS sequence is unique among the species in this study in having an apparent 6 bp insertion (positions 42–47 of the aligned sequence) and a 1 bp deletion (position 124). The two species are divergent, however, for the *rbcL* fragment for 0.7% of the sequence. In contrast to information from morphology and molecules highlighting their similarity, these two species appear to be ecologically allopatric: *A. oahuensis* occurs in the Koolau Mountains of Oahu in wet forests between 300 and 550 m elevation and is epiphytic on a variety of woody plant species, both native and exotic; *A. haalilioanus* is also found on Oahu

but in wet forests above 600 m elevation and most commonly on native species of *Hedyotis* L. (Rubiaceae) (Bishop, 1974; D. Palmer, pers. comm.; personal observations). *Adenophorus haalilioanus* is also common in wet forests on Kauai (K. Wood, pers. comm.). Potentially important distinctions between the two species for the *rbcL* gene are that *A. oahuensis* has one autapomorphic, amino-acid substitution mutation and *A. haalilioanus* has two. Thus, we suggest that these two species have recently diverged from a common ancestor and are maintained on separate evolutionary trajectories via ecological separation.

Another highly supported putative relationship is the sister-taxon status of *A. pinnatifidus* with the *A. oahuensis*–*A. haalilioanus* clade. The long branch supporting this trio of species in the hypothetical phylogeny includes an inferred, synapomorphic amino acid substitution due to a 2nd-codon-position mutation at position 1084 of *rbcL*, inducing a change from glutamine to isoleucine (negatively charged to a nonpolar residue). These three species are unique in the genus in sharing the vegetative-reproductive feature of adventitious root buds. [Root buds are also found in at least one neotropical species of Grammitidaceae (*Melpomene anfractuosa* (Kunze ex Klotzsch) A.R. Sm. & R.C. Moran; Smith and Moran, 1992), however, our larger analysis of 51 species did not support a close relationship between that species and *Adenophorus*. Thus, root buds of *M. anfractuosa* appear to have been derived independently of those found in *Adenophorus*.] *Adenophorus pinnatifidus* is quite distinct morphologically from the other two species in that the leaves are always deeply pinnatifidly lobed, and it is geographically more widespread, occurring on all of the Hawaiian islands. In addition, *A. pinnatifidus* had 17 autapomorphic mutations in our analysis of combined molecular data.

Adenophorus periens is arguably the most enigmatic species in the genus, in terms of morphology. The species has several unique features within the genus, including long, pendulous leaves up to 40 cm or more in length (at least twice as long as any other species in the group), leaf lobes that are twisted out of the plane of the leaf blade, giving a "venetian blind" effect, and the possession of unique, marginal, uniseriate laminar hairs that radiate from leaf margins at a 45–90° angle when fresh. Apparently, Bishop (1974) classified *A. periens* in subg. *Oligadenus* solely based on its pinnatifid leaves. Our analyses of DNA sequence variation strongly support *A. periens* as highly distinct from the remainder of the genus.

4.3. Extant relatives and biogeographical origins of Adenophorus

The phylogenetic placement of *G. tenella* as sister to *Adenophorus* has some interesting implications for morphological evolution within *Adenophorus*. The most obvious character, and one that is often used as a major determinant of generic assignment in ferns, is degree and form of leaf-blade dissection. *Grammitis tenella* has simple, entire, grass-like leaves that are superficially similar to those of *A. oahuensis*. The sister-taxon affinity of *G. tenella*–*Adenophorus* as a group, along with the fact the that *G. bryophila* outgroup clade has simple leaves, implies that simple, entire leaves are ancestral in the Hawaiian group and that more dissected leaves are evolutionarily derived. This evolutionary transition is contrary to the widespread belief that simple leaves have usually been derived from compound leaves in ferns (e.g., Tryon, 1964).

Our molecular data provide robust support for the sister-taxon relationship of the monophyletic *Adenophorus* clade and *G. tenella*, thus supporting a hypothesis that these taxa share a common ancestor that originally colonized the archipelago. *Chrysogrammitis*, the group that had been cited as having similar glandular paraphyses to *Adenophorus* spp. (Parris, 1997, 1998a), was not supported as a close relative in our larger analyses (results to be reported separately). The clade of four species that was strongly supported in our larger analysis of 51 *rbcL* and 51 *atpβ* sequences (not shown) as sister to the *G. tenella*–*Adenophorus* clade (and as represented by *G. bryophila* in this study) comprises three widespread neotropical species and one species from La Réunion (*G. melanoloma*). Thus, we cannot at this time hypothesize a particular geographical origin for the ancestor of the Hawaiian group.

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