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Molecular phylogeny of *Banza* (Orthoptera: Tettigoniidae), the endemic katydids of the Hawaiian Archipelago

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Abstract

The extant endemic katydids (Orthoptera: Tettigoniidae) of the Hawaiian Archipelago include one to three species per high island and a single species on Nihoa, all currently placed in the genus *Banza*. These acoustic insects provide an excellent opportunity for investigating the evolution of reproductive isolation and speciation, but such studies require an understanding of phylogenetic relationships within the group. We use maximum parsimony, likelihood-based Bayesian inference, and maximum likelihood to infer phylogenetic relationships among these taxa, based on ~2 kb of mitochondrial cytochrome oxidase I and cytochrome *b*. Our results strongly support two distinct high island clades: one clade ("Clade I") composed of species from Kauai, Oahu, Molokai, and Lanai and another clade ("Clade II") composed of species from Maui and Hawaii (*Banza unica*, from Oahu, may be basal to both these clades, but its placement is not well resolved). Within these clades, some inferred relationships are strongly supported, such as the sister status of *B. kauaiensis* (Kauai) and *B. parvula* (Oahu) within Clade I, but other relationships remain more ambiguous, such as the relative position of *B. brunnea* (Maui) within Clade II. Although a detailed reconstruction of the historical biogeography of the Hawaiian katydids is difficult, we use our genetic data combined with the known geological history of the Hawaiian Islands to set limits on plausible historical scenarios for diversification of this group. Beyond these historical biogeographic inferences, our results indicate possible cryptic speciation on both Oahu and Hawaii, as well as what may be unusually high average rates of nucleotide substitution. The present work sets the stage for future genetic and experimental investigations of this group.

Keywords: *Banza*; Copiphorinae; Tettigoniidae; Hawaii

1. Introduction

The Hawaiian Islands have provided evolutionary biologists with extraordinary opportunities to investigate processes of ecological diversification, evolutionary divergence, and speciation. This group of islands is extremely remote, and natural colonization from continental or other island sources has consequently been extremely rare. In addition to their geographic isolation, the relatively well-known geological history of the Hawaiian Islands makes them particularly well suited for studies of diversification and speciation. The islands comprising the Hawaiian Archipelago were formed successively over a fixed "hot spot" beneath the northwestward-moving Pacific tectonic plate. Estimated dates for the origin of each island (or volcano) are available based on K–Ar dating (Fig. 1).

The biogeography and patterns of speciation of several groups of plants and animals have been the subject of intense study (e.g., Otte, 1994; Wagner and Funk, 1995; Givnish and Sytsma, 1997; Roderick and Gillespie, 1998; Mendelson and Shaw, 2005), especially with respect to dazzling ecological radiations of taxa descended from a

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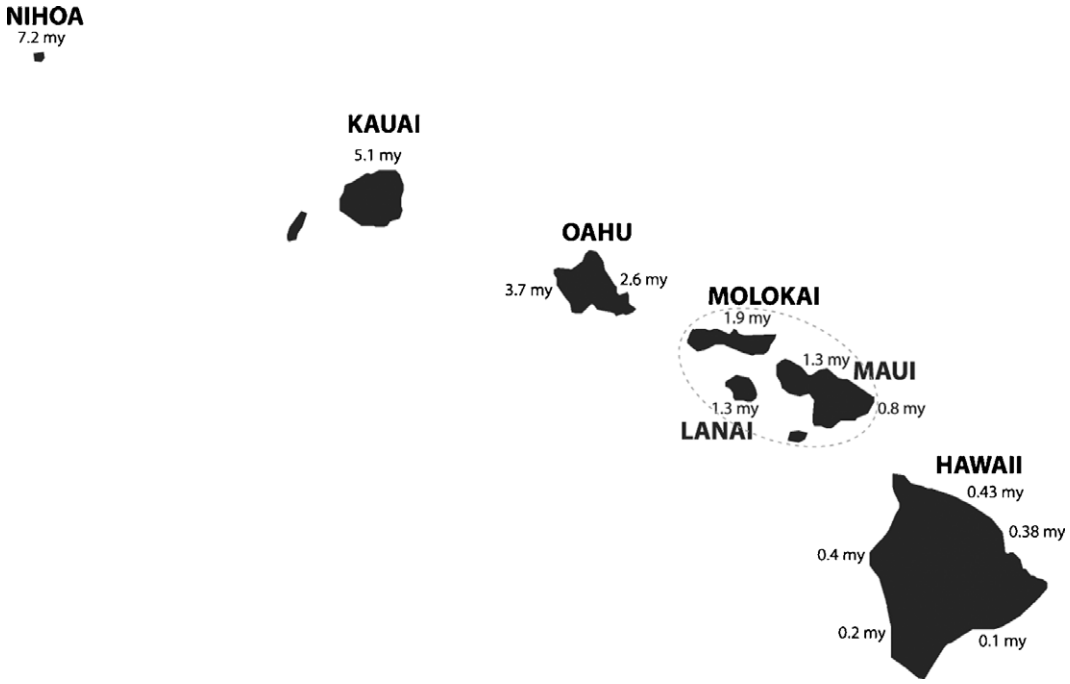

Fig. 1. The main Hawaiian Islands, with approximate ages indicated. Molokai and Lanai were joined as part of Maui Nui ("Greater Maui") until about 0.1 Myr ago (Ma), well after their mutual separation from Maui around 0.3 Ma and the emergence of the Big Island (Hawaii) around 0.5 Ma. The distances between many of the Hawaiian Islands may at one time have been significantly smaller than they are today, facilitating migration between islands. For example, the channel between Kauai and Oahu was apparently far narrower shortly after the emergence of Oahu than it is today and the distance between Maui and Hawaii is now nearly four times what it was 0.37 Ma (Carson and Clague, 1995).

single or very few colonizing ancestors (e.g., silverswords, *Drosophila* flies, honeycreepers). Far less attention has been paid to more modest, apparently "nonadaptive" radiations such as that of the endemic Hawaiian katydids in the genus *Banza*. Comparing patterns of speciation and molecular, morphological, physiological, and behavioral divergence emerging from studies of *Banza* with those documented for other groups of organisms in the Hawaiian Islands will provide a broader perspective on speciation in Hawaii.

The genus *Banza* is composed of a group of fairly small (body length ~15–25 mm), flightless cone-headed katydids in the tribe Copiphorini within the subfamily Conocephalinae *sensu lato* (Naskrecki and Otte, 1999). [Note that general comments made here regarding *Banza* do not necessarily apply to *Banza nihoa*, which is probably improperly assigned to this genus (Strazanac, 1996); see Section 4.] *Banza* are quite distinct morphologically and are found only in wet forests on the main high islands of the Hawaiian Archipelago, generally below ~1200 m (Strazanac, 1996). A summary of the distribution of the endemic Hawaiian katydids is presented in Table 1. With the exception of the two Oahu species and *B. nihoa* (which is found only on Nihoa, far to the northwest of the current high islands), they are limited to forests dominated by native vegetation (Strazanac, 1996).

Banza are acoustic insects. Males rapidly rub together specialized structures on their forewings to produce high-pitched, species-specific songs to which females are attracted. Like most katydid songs, those of *Banza* are very broadband signals, in contrast to the typically pure tones of crickets. The songs of *Banza* consist of a series of rapidly delivered pulses with much of the energy in the ultrasonic, ranging from ~15 to 40 kHz (Strazanac, 1996). The importance of acoustic signals in *Banza* reproductive behavior suggests numerous avenues of investigation of acoustic evolution and evolution of reproductive isolation among *Banza* species both within and among islands, but such studies require a phylogenetic context for meaningful interpretation.

Table 1
Distribution of endemic Hawaiian katydids (data compiled from Strazanac, 1996)

Island	Species
Nihoa	"*Banza nihoa*" ("*Conocephaloides nihoa*")[a]
Several?	*Conocephaloides remotus* (extinct)
Kauai	*Banza kauaiensis*[b]
	"*B.k. kauaiensis*" (east of Waimea Canyon; short-winged)
	"*B.k. affinis*" (west + north of Waimea Canyon; long-winged)
Oahu	*Banza unica*
	Banza parvula
Molokai	*Banza molokaiensis*
Lanai	*Banza deplanata*
Maui	*Banza brunnea* (central + western West Maui)
	Banza mauiensis (eastern West Maui)
	"*Banza pilimauiensis*" n. sp. (East Maui + possibly eastern West Maui)[c]
Hawaii	*Banza nitida*

[a] Probably does not actually belong in either *Banza* or *Conocephaloides* (see text).
[b] Two forms not yet formally demoted to subspecies.
[c] Not yet formally described.

Little is known about *Banza* relationships. Although the extant sister group to *Banza* is not confidently known, the genus appears to be closely related to the New World copiphorines *Neoconocephalus* (e.g., Dadour and Bailey, 1990; Greenfield, 1990) and the Old World copiphorines *Euconocephalus* and *Ruspolia*, which in their current formulation are indistinguishable from *Neoconocephalus* except by geography (Strazanac, 1996; Naskrecki, 2000). In stark contrast to the very diverse Hawaiian crickets (Otte, 1994; Shaw, 2000), the known native katydid fauna from the Hawaiian Archipelago includes few species, all apparently derived from between one and three independent colonizations (Strazanac, 1996). The genus *Banza* has traditionally included all the endemic katydids of Hawaii, with the exception of the apparently extinct *Conocephaloides remotus*, which may actually have represented a species complex (Strazanac, 1996). Although *B. nihoa* has traditionally been placed in the genus *Banza*, Strazanac (1996) presented evidence suggesting that this species may in fact belong in *Conocephaloides* or represent an independent Hawaiian lineage. Strazanac undertook a phylogenetic analysis of *Banza* using morphological and behavioral characters with limited success, mainly due to the extremely limited number of phylogenetically useful morphological characters (Strazanac, 1996).

In the present study, we use DNA sequences from the mitochondrial genes cytochrome oxidase I (COI) and cytochrome *b* to reconstruct the phylogenetic relationships among all extant *Banza* (although data from nuclear genes would be desirable as well, these could not be obtained from the type of material available to us, namely eggs). Our results provide a phylogenetic context for future studies of this group and begin to clarify some of the taxonomic issues raised by Strazanac (1996).

2. Materials and methods

2.1. Sampling

In the course of his investigations of the systematics and acoustic behavior of *Banza*, Strazanac (1996, in preparation) collected all the recognized *Banza* taxa and reared nearly all of them in the lab. For all *Banza* DNA extractions, we used eggs obtained by Strazanac from field-collected or lab-reared females (see Appendix A for collection localities). These eggs were stored in 95–100% ethanol between the time they were collected (between 1987 and 1993) and the time they were extracted in 2000 and 2001; during most of this period, they were stored at ~4 °C. As our outgroups, we used *Orocharis saltator* (an eneopterine cricket), *Orchelimum nigripes* (a conocephaline katydid), and *Neoconocephalus triops* (a copiphorine katydid). Our DNA source for each of these was a hind femur from a specimen kept frozen at −80 °C. For each ingroup species, we extracted DNA from eggs of at least two females (eggs from different individuals were processed at different times).

2.2. Genetic analysis

DNA was extracted using Qiagen DNeasy Tissue Kits using several pooled eggs per female. Sequences of primers used are given in Table 2. To amplify approximately the first half of COI, we used two primers published by Folmer et al. (1994), LCO1490 and HCO2198; this primer pair amplifies a 658-bp region of COI (excluding primers). To amplify approximately the second half of COI, we used various combinations of two primers designed by Bely and Wray (2004), COI-A^{+} and COI-B^{-}, as well as two versions of these primers modified for the present study, COI-A^{+}GR and COI-B^{-}GR; these primers amplify a region of COI of 679 bp (excluding primers). In total, we obtained 1255 bp of usable COI sequence common to most individuals in our data set.

For cytochrome *b* amplification, we used primers CytbP21 and Cytb2R (Huang et al., 2000) and two versions of these primers modified for the present study, CytbP21b and Cytb2Ra. These primers amplify a region of 729 or 748 bp (excluding primers), depending on the primer pair, and yielded 748 bp of usable sequence common to most individuals in our data set.

For most COI amplifications, we used 35 cycles of 94 °C (30 s)–50 °C (30 s)–72 °C (50 s). For most cytochrome *b* amplifications, we used 35 cycles of 94 °C (30 s)–47 °C (30 s)–72 °C (50 s). PCR products were cleaned using Qiagen QIAquick PCR Purification Kits and cycle sequenced in both directions using an ABI PRISM cycle sequencing kit. Sequenced products were cleaned using Princeton Separations CentriSep columns and run out on an ABI 377 automated sequencer.

2.3. Phylogenetic analysis

We aligned and edited our sequences [a total of 2003 (1255 + 748) nucleotide positions, 722 of which were variable] using Sequencher 3.1.1 (Genecodes Corporation, Ann Arbor, MI). We then used three approaches to infer phylogenetic relationships among our taxa: maximum parsimony, likelihood-based Bayesian inference, and maximum likelihood.

Table 2
Primers used in this study for cytochrome oxidase I (COI) and cytochrome *b* (Cyt-*b*)

Gene	Primer name	Sequence (5′ to 3′)
CO I	LCO1490[a]	GGTCAACAAATCATAAAGATATTGG
CO I	HCO2198[a]	TAAACTTCAGGGTGACCAAAAAATCA
CO I	COI-A^{+}[b]	CCTGTTCTTGCTGGTGCTATTACNAT
CO I	COI-B^{-}[b]	TAGTCAGAATATCGCCGAGGTATNCC
CO I	COI-A^{+}GR	CCAGTTTTAGCTGGTGCTATTACIAT
CO I	COI-B^{-}GR	TAATCRGAATATSGTCGTGGTATTCC
Cyt-*b*	CytbP21[c]	CCATCCAACATCTCAGCATGATGAAA
Cyt-*b*	Cytb2R[c]	CCWARTTTATTAGGAATAGATCG
Cyt-*b*	CytbP21b	TGATGAAAYTTIGGTTCICTIITAGGA
Cyt-*b*	Cytb2Ra	ACTCCTCCTAATTTATTAGGIATIGATCG

[a] Folmer et al. (1994).
[b] Bely and Wray (2004).
[c] Huang et al. (2000).

2.3.1. Maximum parsimony

For our maximum parsimony (MP) analysis, we assessed whether combining the COI and cytochrome *b* data was warranted using the partition homogeneity test implemented in PAUP*, version 4.0b10 for Macintosh (Swofford, 2002). This test gave no indication of significant heterogeneity. Perhaps more important, given the low power of this test under some conditions (Darlu and Lecointre, 2002), when analyzed separately COI and cytochrome *b* yielded essentially congruent trees with no well-supported differences. Thus, the analyses presented here are on the combined data set of 2003 nucleotide positions. Parsimony analyses were performed using the heuristic search option with 20 random addition replicates (other settings used were PAUP* defaults; a branch-and-bound search yielded the same MP tree). Node support was estimated using nonparametric bootstrapping (1000 pseudoreplicates with 20 random additions each). In addition, we calculated Bremer support indices (Bremer, 1988) for each node using TREEROT, version 2 (Sorenson, 1999).

2.3.2. Bayesian inference

For our Bayesian likelihood analysis (BA), we used MrBayes, version 2.01 (Huelsenbeck and Ronquist, 2001), which implements a Markov chain Monte Carlo approach to approximate the posterior probabilities of phylogenetic trees and clades. We selected our basic model using MrModeltest, version 1.1b (Nylander, 2002), which utilizes hierarchical likelihood ratio tests to select a parameterized model that best fits the data on a neighbor-joining tree. The basic model selected for this analysis (GTR + I + Γ) assumes a general time-reversible (GTR) relative rate matrix (i.e., six-parameter R-matrix), a fraction of sites that are invariant (I), and a parameter describing a gamma distribution of rates of change for evolving sites (Γ). Analyses were also performed using two other models of sequence evolution: (1) a two-parameter HKY model (Hasegawa et al., 1985), with relative rates estimated for each codon position ("HKY + site-specific") and (2) a GTR model with relative rates estimated for each codon position ("GTR + site-specific"). All three of our models produced identical tree topologies and, with just a few exceptions noted below, nearly identical clade credibility values. Each run was based on a chain of 10^6 generations (along with three heated chains to facilitate a more efficient search of tree space), with a tree sampled every 100 generations. We examined plots of tree likelihood versus generation number to determine when a chain appeared to have reached stationarity (and was therefore sampling trees approximately according to their posterior probabilities); to be conservative, we treated considerably more than this number of trees as our "burn-in", i.e., trees to be excluded from the analysis. For each run, we then computed a consensus tree based on 7000 trees (10,001 total trees—3001 burn-in trees), with the estimated posterior probability of each clade being the proportion of sampled trees in which it occurred. For each model examined, we compared results from three independent runs, each initiated with a random tree, in order to minimize the possibility of identifying local rather than global optima.

2.3.3. Maximum likelihood

Maximum likelihood (ML) analyses were performed for the GTR + I + Γ model, with parameter values previously estimated by MrModeltest, using the heuristic search option with 20 random additions in PAUP*, version 4.0b10 (other settings were PAUP defaults). Node support was estimated using nonparametric bootstrapping (100 pseudoreplicates, with five random additions each). We also performed ML analyses for our "HKY + site-specific" and "GTR + site-specific" models by estimating parameter values on a neighbor-joining tree, then using these values in a heuristic search as just described above.

3. Results

3.1. Genetic distances

Sequences used in this study have been deposited in GenBank (for GenBank accession numbers, see Appendix A).

Table 3
Interspecific genetic distances (combined COI + Cyt *b*, uncorrected) among *Banza* species and three outgroups (two other katydids, *Neoconocephalus* and *Orchelimum*, and a cricket, *Orocharis*)

	Oroch.	*Orchel.*	*Neocon.*	*nihoa*	*kauai.*	*parv.*	*unica*	*depl.*	*molok.*	*brun.*	*maui.*	*"pili."*	*nitida*
Orocharis	—												
Orchelimum	0.202	—											
Neoconocephalus	0.195	0.174	—										
Banza nihoa	0.198	0.166	0.159	—									
B. kauaiensis	0.193	0.188	0.153	0.142	—								
B. parvula	0.194	0.189	0.156	0.137	0.046	—							
B. unica	0.202	0.181	0.151	0.142	0.098	0.094	—						
B. deplanata	0.195	0.197	0.151	0.145	0.073	0.078	0.097	—					
B. molokaiensis	0.199	0.197	0.158	0.138	0.064	0.064	0.092	0.062	—				
B. brunnea	0.197	0.197	0.154	0.146	0.089	0.092	0.089	0.085	0.085	—			
B. mauiensis	0.195	0.191	0.150	0.138	0.089	0.093	0.082	0.081	0.086	0.049	—		
B. "pilimauiensis"	0.197	0.194	0.151	0.138	0.089	0.092	0.083	0.083	0.085	0.050	0.011	—	
B. nitida	0.202	0.192	0.154	0.144	0.095	0.096	0.093	0.088	0.090	0.051	0.052	0.054	—

Individuals used are all (A) of Figs. 2 and 3.

Table 4
Intraspecific genetic distances (COI + Cyt-*b*, uncorrected) between *Banza* individuals

Conspecific individuals	Genetic distance
nihoa A–*nihoa* B	0.000
kauaiensis A–*kauaiensis* B	0.010
parvula A–*parvula* B	0.013
unica A–*unica* B	0.039
deplanata A–*deplanata* B	0.000
molokaiensis A–*molokaiensis* B	0.001
brunnea A–*brunnea* B	0.003
mauiensis A–*mauiensis* B	0.000
"*pilimauaiensis*" A–"*pilimauiensis*" B	0.003
nitida A–*nitida* B	0.017
nitida A–*nitida* C	0.053
nitida B–*nitida* C	0.049

Uncorrected interspecific and intraspecific genetic distances are given in Tables 3 and 4. Intraspecific distances were 1% or less (but note that some of these conspecifics were from the same population, see Appendix A), with two notable exceptions: (1) our *unica* sequences from Oahu's Wai'anae and Ko'olau Mountain ranges were 3.9% divergent and (2) two of our three *nitida* sequences were 1.7% divergent, while the third was about 5% divergent from the other two.

3.2. Parsimony analysis

Of the 722 variable sites, 537 (74%) were parsimony informative. Our single most parsimonious tree had a length of 1711, a consistency index of 0.584, a retention index of 0.696, and thus a rescaled consistency index of 0.406. All conspecific individuals (with the exception of *nitida*, see below) were grouped with 100% bootstrap support (Fig. 2). A bootstrapped neighbor-joining (NJ) analysis yielded a topology identical to the MP tree except that the positions of *brunnea* and *nitida* were swapped (as in all our analyses, however, support for the relative placement of these two taxa was extremely weak, with bootstrap support of just 51% from 1000 pseudoreplicates).

3.3. Bayesian analysis

Within each of the three models explored in our Bayesian analysis, the three replicate MrBayes runs yielded identical tree topologies and nearly identical clade credibility values. Furthermore, all three models yielded identical tree topologies, and all intraspecific groupings (including *nitida*) had associated clade credibility values of $\geqslant 0.99$ (Fig. 3).

There were no well-supported conflicts between the MP and Bayesian analyses. Results from the Bayesian analysis supported all of the strongly supported clades recovered in the parsimony analysis, as well as several additional nodes (Figs. 2 and 3). *Banza nihoa* is basal to all other *Banza*. Two strongly supported clades within the non-*nihoa Banza* were recovered by the Bayesian analysis. One clade ("Clade I") is composed of *kauaiensis* (Kauai), *parvula* (Oahu), *molokai-*

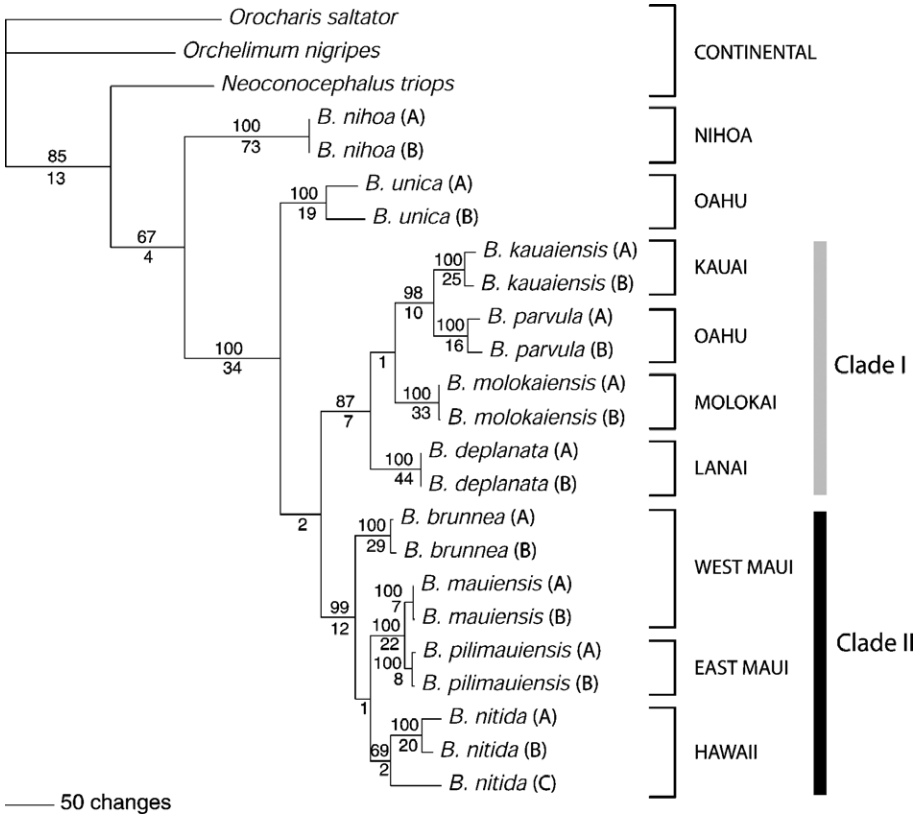

Fig. 2. Maximum parsimony tree. Bootstrap support greater than 50% (based on 1000 pseudoreplicates) is indicated above each branch; Bremer support (decay index) is indicated below each branch.

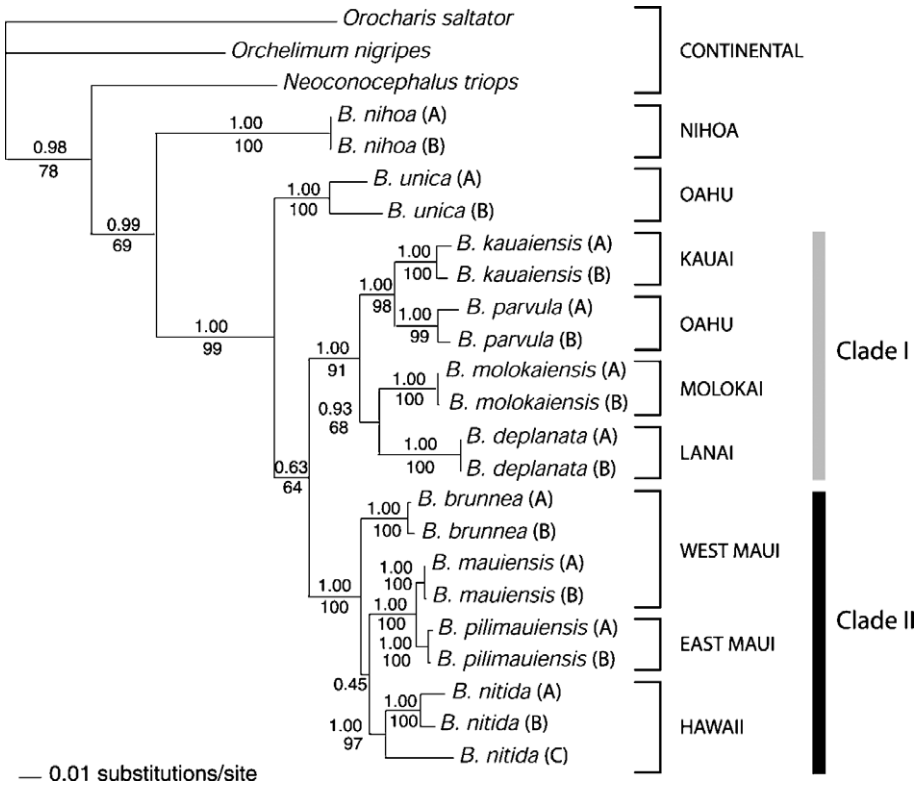

Fig. 3. Tree generated by Bayesian analysis using MrBayes, assuming GTR + I + Γ model, with clade credibility values shown above branches. This tree was the result of one of the three replicate runs using this model, all of which yielded identical tree topologies and nearly identical clade credibility values. All 20 replicates from a maximum likelihood analysis assuming a GTR + I + Γ model (see text) yielded the same tree topology as the Bayesian analysis. Maximum likelihood bootstrap support greater than 50% (based on 100 pseudoreplicates) is indicated below each branch.

ensis (Molokai), and *deplanata* (Lanai), and the other ("Clade II") is composed of *brunnea* (West Maui), *mauiensis* (West Maui), "*pilimauiensis*" (East Maui), and *nitida* (Hawaii). Depending on the model used (see below), the Bayesian analysis either strongly or weakly suggests that *unica* is an outgroup to all other high island (i.e., non-*nihoa*) *Banza*. This same topology was recovered in our MP tree, but bootstrapping indicates that with MP this placement of *unica* with respect to Clades I and II is very poorly supported. Within Clades I and II, both MP and Bayesian analyses recover as sister taxa (1) *kauaienis* and *parvula* and (2) *mauiensis* and "*pilimauiensis*". Although the bootstrapped MP tree does not resolve the relative placement of *deplanata* within Clade I, the Bayesian analysis indicates that *deplanata* is sister to *molokaiensis*, with this pair in turn sister to (*kauaiensis* + *parvula*). Neither MP nor the Bayesian analysis clearly resolves the relative positions of *brunnea* and *nitida* within Clade II.

For all three models examined in our Bayesian analysis, all nodes were strongly supported (>0.95), with the following three exceptions: (1) the placement of *unica* as an outgroup to all other non-*nihoa Banza* had a clade credibility value of $\geqslant 0.98$ in models "GTR + site-specific" and "HKY + site-specific," but only about 0.6 in model "GTR + I + Γ"; (2) the clade (*deplanata* + *molokaiensis*) had a clade credibility value of $\geqslant 0.98$ with model "GTR + site-specific," $\geqslant 0.94$ with model "HKY + site-specific," and only $\geqslant 0.90$ with model "GTR + I + Γ"; (3) the placement of *brunnea* as basal in the clade (*mauiensis* + "*pilimauiensis*" + *nitida* + *brunnea*) was indicated by all three models (as it was also in the MP tree), but support was extremely weak, with a clade credibility value of about 0.74 in model "GTR + site-specific," 0.61 in model "HKY + site-specific," and 0.45 in model "GTR + I + Γ."

3.4. Maximum likelihood analysis

For "GTR + I + Γ," all 20 random addition replicates in the ML analysis produced the same topology, which was identical to that recovered by the Bayesian analysis (Fig. 3). Furthermore, bootstrap support for all nodes of interest was very high, with three notable exceptions. First, the placement of *unica* as basal to all other non-*nihoa Banza* had bootstrap support of only 64% (as compared with 0.58/0.63/0.63 clade credibility in the three replicate BA runs using "GTR + I + Γ," and <50% bootstrap support for MP; as noted above, BA using "GTR + site-specific" or "HKY + site-specific" yielded clade credibility values for this placement of *unica* of $\geqslant 0.98$). Second, although *deplanata* and *molokaiensis* were recovered as sister taxa in this analysis (as they were also in the ML analyses using the "GTR + site-specific" and "HKY + site-specific" models, data not shown, and in all three models examined in the Bayesian analysis), bootstrap support for this grouping was

only 68%. Finally, the relative positions of *brunnea*, (*mauiensis* + "*pilimauiensis*"), and *nitida* were not clearly resolved in the ML analysis, as was also the case for both the MP and Bayesian analyses. However, although clade support for this part of the tree, as measured by bootstrapping (MP and ML) or estimated prior probabilities (Bayesian analysis), was very weak, all three methods nevertheless recovered the same topology, placing *brunnea* basal to the other two clades, with the exception of ML "HKY + site-specific," which placed *nitida* basal to *brunnea* and (*mauiensis* + "*pilimauiensis*"). Except for the different placement of *brunnea* relative to (*mauiensis* + "*pilimauiensis*") and *nitida* in the ML "HKY + site-specific" analysis, ML searches (not bootstrapped) using alternative models "GTR + site-specific" and "HKY + site-specific" yielded tree topologies identical to those found in our BA (all three models) and "GTR + I + Γ" ML analyses.

4. Discussion

The close similarity of trees obtained using three different methods of analysis—maximum parsimony, likelihood-based Bayesian inference, and maximum likelihood—and using several different models for the two likelihood-based methods suggests that our phylogenetic estimates are robust. The one major uncertainty in our reconstruction is the position of *unica*. There was some inconsistency among our various analyses in how strongly the basal placement of *unica* was supported. The placement of *unica* as basal to all other non-*nihoa Banza* was weakly ("GTR + I + Γ") or very strongly ("GTR + site-specific" and "HKY + site specific") supported in our Bayesian analysis, depending on the model used; weakly supported in our bootstrapped maximum likelihood analysis ("GTR + I + Γ"); and unresolved in our bootstrapped maximum parsimony analysis. The fact that the basal placement of *unica* was recovered in the MP tree, was recovered in the ML trees using all three models examined, and was favored in consensus trees using all three BA models examined is certainly noteworthy, but given the weak support for this placement in most of our analyses this question must be investigated further with additional data. Interestingly, support for this basal placement of *unica* was 0.58/0.63/0.63 in the three replicate "GTR + I + Γ" BA runs versus 64% in the bootstrapped "GTR + I + Γ" ML analysis. Thus, although recent work has indicated that currently implemented Bayesian methods of phylogenetic inference may tend to overestimate clade support under some conditions (e.g., Suzuki et al., 2002; Cummings et al., 2003; Douady et al., 2003; Pickett and Randle, 2005; but see, e.g., Alfaro et al., 2003), clade credibility values from our Bayesian analysis and bootstrap percentages from our ML analysis matched each other closely in supporting (very weakly) this placement of *unica*. Several authors have shown that substitution model misspecification can strongly bias BA probability estimates, often yielding inappropriately high numbers, and that this problem may tend to be especially serious in the case of overly simple models (e.g., Suzuki et al., 2002; Erixon et al., 2003; Huelsenbeck and Rannala, 2004; Lemmon and Moriarty, 2004). It is possible that the placement of *unica*—which had a clade credibility value of $\geqslant 0.98$ in models "GTR + site-specific" and "HKY + site-specific," but only about 0.6 in model "GTR + I + Γ"—provides an empirical example of this phenomenon. The placement of *brunnea* may provide a similar example (see above). For the most part, however, clades of interest received either strong (most nodes) or weak [placement of *brunnea* relative to (*mauiensis* + "*pilimauiensis*") and *nitida*] support in both the Bayesian analysis and bootstrapped ML analysis.

4.1. Biogeographic history

Assuming the placement of *unica* in our analyses is correct, a parsimonious mapping of islands on the *Banza* phylogeny indicates that the common ancestor of all extant non-*nihoa Banza* probably lived on Oahu, with a colonization of Kauai and a colonization of Maui Nui in Clade I, and in Clade II an independent colonization of Maui Nui with a subsequent colonization of Hawaii from Maui. Although it is difficult at best to infer in detail the history of intra-island speciation and extinction and inter-island dispersal, we can nevertheless draw a number of conclusions regarding the possible timing and geography of *Banza* diversification by examining genetic distances in the context of the known geological history of the Hawaiian Islands. Our results [see (5), (6), and (7)] suggest a repeated pattern of colonization of new islands followed by extinction of the colonizing lineage on the source island and/or unusually high mtDNA substitution rates, on the order of at least 3–4% per Myr. Our data suggest the following seven specific conclusions:

1. The original colonists may have colonized Oahu or an older island.

Strazanac (1996) argued that morphological, acoustic, behavioral, and ecological data all suggest that *B. nihoa* does *not* fall within a monophyletic *Banza* clade, meaning true *Banza* occur only on Kauai and the younger islands. This hypothesis is consistent with the general conclusion by Price and Clague (2002), based on their analysis of landscape changes in the Hawaiian Archipelago over the last 32 Myr, that contemporary species living in montane habitats probably either arrived from outside the Hawaiian Archipelago or evolved within the archipelago after the formation of Kauai. Unfortunately, our genetic data cannot address the question of *Banza* monophyly in the absence of extensive sampling of other Pacific island and mainland copiphorines. However, if, in contrast to Strazanac's conclusion, *nihoa is* in fact the basal representative of a monophyletic *Banza* clade, we can estimate the divergence time between *nihoa* and the other extant *Banza* taxa using an approximate average mtDNA divergence rate of 2.3% divergence per Myr, as has been estimated for several

groups of arthropods (Brower, 1994; for very similar estimates for vertebrates, see Brown et al., 1979; Klicka and Zink, 1997, 1998; Fleischer et al., 1998). The (uncorrected) genetic distances between *nihoa* and the other *Banza* taxa, which range from ~13 to 15% (Table 3), would suggest a divergence time of about 5.6–6.5 Ma, which would be consistent with the age of Nihoa, although older than the ages of any of the current high islands (Fig. 1). Thus, in this scenario Nihoa is the likely site of the original colonization of the archipelago by the ancestor of the extant *Banza*. Alternatively, if *nihoa* actually represents an independent (non-*Banza*) colonization event, as suggested by Strazanac, which islands are possible candidates as home to the common ancestor of all extant *Banza*? All of our phylogenetic analyses (NJ, MP, BA, ML) placed *unica* as basal to all other non-*nihoa Banza* (although this placement was not generally well supported). The average genetic distance between *unica* and all these other taxa was 9.2%. This would suggest a divergence time between *unica* and the other taxa of about 4 Ma, younger than Kauai but slightly older than the oldest K–Ar age estimates for Oahu (Fig. 1). However, if we allow for a slightly faster average sequence divergence rate (e.g., 2.5% or more per Myr), we conclude that the ancestor of the extant non-*nihoa Banza* could have colonized Kauai or Oahu (but not any of the younger islands). Based on average genetic distances between taxa in Clades I and II, this conclusion holds even if *unica* actually falls within one of these clades.

2. The common ancestor of Clades I and II occurred on Oahu or an older island.

Average divergence between taxa in Clades I and II was about 8.6%, implying a common ancestor for these two clades dating back about 3.7 Ma. This suggests that the ancestor of these two clades must have occurred on Oahu or an older island.

3. The common ancestor of Clade I taxa occurred on Kauai or Oahu.

The common ancestor of (*kauaiensis* + *parvula*) and (*molokaiensis* + *deplanata*), based on an average divergence between these two clades of 6.6%, dates to an estimated 2.9 Ma, consistent with the ages of both Kauai and Oahu, but not Maui Nui. Thus, the common ancestor of the Clade I taxa probably occurred on Kauai or Oahu.

4. The common ancestor of Clade II taxa occurred on Maui Nui or an older island.

Depending on the relative positions of *brunnea* and *nitida* in Clade II, which are not confidently resolved in any of our analyses (although MP, ML, and BA all indicate that *brunnea* is basal in this clade), average divergence among taxa within this clade is about 5.1 or 5.4%, which implies an estimated divergence time of about 2.2–2.3 Ma. This is older than the oldest geological date for the Maui Nui complex (on current day Molokai), but if we allow for a slightly faster divergence rate (e.g., 2.7% or more per Myr) then we conclude that the Clade II ancestor could have occurred on Maui Nui or any older island (Fig. 1).

5. The common ancestors of (a) *kauaiensis* and *parvula* and of (b) *molokaiensis* and *deplanata* occurred on Maui Nui or an older island.

Within Clade I (Figs. 2 and 3), *kauaiensis* and *parvula* showed an average sequence divergence of 4.3%. This yields an estimated divergence time of about 1.9 Ma, consistent with a common ancestor on Kauai, Oahu, or Maui Nui (Fig. 1). Remarkably, *molokaiensis* and *deplanata* showed a divergence of 6.1%, yielding an estimated divergence time of about 2.6 Ma, consistent with a common ancestor on Kauai or Oahu, but probably not on Maui Nui, although Maui Nui ancestry might be plausible if the average sequence divergence rate were around 3% per Myr or faster (Fig. 1), which would be consistent with tentative conclusions about substitution rates in (6) and (7). If the ancestor lived on Oahu or Kauai rather than Maui Nui, this would imply an extinction of this lineage on the source island subsequent to colonization of Maui Nui. In any case, the deep divergence between *molokaiensis* and *deplanata* certainly indicates that their divergence long pre-dates the separation of present-day Molokai and Lanai ~0.1 Ma, as well as the isolation of current-day Maui ~0.3 Ma.

6. All three *Banza* species on Maui probably diverged within the island.

Among *Banza* currently known from Maui, genetic distances range from 1.1% (*mauiensis*–"*pilimauiensis*"), which implies a divergence time of less than 0.5 Ma (entirely plausible given Maui's age of ~1.3 Myr), to 4.9% (*brunnea*–"*pilimauiensis*"), which implies a divergence time of >2 Ma. This latter divergence time estimate is older than Maui itself, but if the actual average substitution rate is 3.8% per Myr or greater, an intra-island origin for the (Maui + Hawaii) clade is possible. If the ancestor lived on an older island, rather than on Maui, this would imply an extinction of this lineage on the source island subsequent to colonization of Maui. Interestingly, genetic distances among Hawaii samples suggest an average mtDNA substitution rate for Maui *Banza* of ~4% per Myr or more [see (7)], and a possible substitution rate of at least ~3% per Myr is suggested in (5).

7. *Banza unica* and *B. nitida* may include cryptic species. Two *unica* individuals collected on Oahu from different mountain ranges (the Wai'anae and Ko'olau Mountains) exhibited a genetic distance of 3.9%, implying a divergence time of about 1.7 Ma. Such a high level of sequence divergence suggests that the taxon known as *B. unica* may in fact include cryptic species. Investigation of this question will require further genetic sampling of *unica* from these two ranges, as well as detailed analyses of songs and morphology and experimental studies of reproductive isolation.

From the island of Hawaii, we obtained sequence from three *nitida* individuals, one each from Kealakekua (west side of Hawaii), Stainback (east side of Hawaii), and Puu Iki (north side of Hawaii in the Kohala Mountains, the oldest portion of the island; see Appendix A). We found that the first two were about 1.7% divergent, while the third (from Puu Iki) was a remarkable 5.3 and 4.9% divergent, respectively, from the other two. At 2.3% divergence per

Myr, these genetic distances would imply divergence times of about 0.7, 2.3, and 2.1 Ma. Given Hawaii's age of less than 0.5 Myr, such deep divergences suggest that (a) substitution rates are greater than 2.3% per Myr (1.7% per 0.5 Myr implies a minimum rate of at least 3.4% per Myr) and (b) the highly divergent individual from the Kohala Mountains may represent a cryptic species associated with either an extraordinarily accelerated substitution rate of ~10% per Myr (which seems implausible) or an independent colonization of the island from an older island (which seems more likely). The three *nitida* individuals grouped together in all our analyses, very strongly supported in the Bayesian analysis (1.00 clade credibility) and ML (97% bootstrap support) and moderately supported in MP (69% bootstrap support, Bremer support of 2). Thus, it seems likely that Hawaii was colonized at least twice from Maui by representatives of a Maui "*nitida* group," all of which are now extinct on Maui [an island source older than Maui is far less plausible given the very strong support in all our analyses for a monophyletic clade including all the extant taxa on (Maui + Hawaii)]. Given Maui's estimated age of 1.3 Myr, this scenario would imply a rate of "*nitida* group" sequence evolution of at least ~4% per Myr. Further sampling and analyses will be necessary to clarify this intriguing problem.

4.2. Taxonomic issues

Concepts of species boundaries in *Banza* have been somewhat fluid during the past 150 years. The treatments by Perkins (1899, 1910) and Zimmerman (1948) brought some stability to the situation. However, more recently Strazanac (1996) put forth several important proposals regarding our understanding of *Banza* diversity. Thoroughly addressing all these proposals will require much additional sampling and direct studies of reproductive isolation among *Banza* taxa, but our genetic data support or are at least consistent with several of these hypotheses. For example, Strazanac suggested that *kauaiensis* and *parvula* are sister taxa, based on morphological similarities and on the fact that only these two taxa consistently pair their wingstrokes when singing. This hypothesis is strongly supported by our phylogenetic analysis (Figs. 2 and 3) [interestingly, *Banza deplanata* also occasionally pairs its wingstrokes and our analysis indicates that the (*deplanata* + *molokaiensis*) clade is sister to the (*kauaiensi* + *parvula*) clade]. Genetic support for some other proposals is less clear. Strazanac's suggestion that *B. nihoa* is not part of a monophyletic *Banza* clade cannot be addressed using molecular data without extensive sampling of mainland and Pacific island copiphorines. Our genetic data are not obviously inconsistent with either the traditional view that *nihoa* is simply the most basal extant *Banza* or with Strazanac's hypothesis of an independent origin for *nihoa*. Several of Strazanac's proposals must be investigated further by mating and breeding studies and experimental analyses of patterns of reproductive isolation. For example, Strazanac saw no justification for granting specific status to the long-winged and short-winged forms of *B. kauaiensis*. In our data set, we found a 1% divergence between these two forms, similar to the divergence between our two *parvula* individuals and less than that between our two *unica* or among our three *nitida* (as discussed above, however, *nitida* and *unica* as currently recognized may in fact each represent two or more cryptic species). Thus, these data may support Strazanac's proposal to treat the two forms of *kauaiensis* as a single species with two subspecies. On the other hand, based primarily on very strong acoustic evidence, Strazanac (1996) recognized a new cryptic species (not yet formally described) closely allied to *B. mauiensis*, *B.* "*pilimauiensis*", and we find that sequences from these two sibling species differ by only about 1%, the same distance separating the two forms of *kauaiensis*. Clearly, experimental work will be necessary to clarify the status of the two *kauaiensis* taxa with respect to reproductive isolation. Another question requiring detailed study is the nature of the relationship between *B. parvula* and *B. unica*, the only two sympatric *Banza* taxa. Strazanac (1986, 1996) found that the progeny of a wild-inseminated *parvula* female exhibited a surprising degree of morphological variation. Most of these offspring showed a combination of *parvula* and *unica* characters, but several individuals resembled typical *parvula* or, most notably, *unica*. Strazanac (1986) suggested that either (1) *parvula* and *unica* belong to a single highly variable species or (2) *parvula* and *unica* are two valid species that hybridize to some degree in the wild. Our analysis indicates that *parvula* and *unica* are not even sister taxa and, in fact, appear not to be particularly close at all, clearly favoring the hypothesis of hybridization. Understanding the relationship and interactions between *parvula* and *unica* will require extensive sampling, with analysis of morphology and both mitochondrial and nuclear genotypes as well as mating and breeding trials.

Among Strazanac's most intriguing proposals was that *B. nitida* may vary clinally around the perimeter of the island of Hawaii. He noted that *nitida* is distributed around the perimeter of the island, with the only known wide break located between Hualalai Mountain and the Kohala Mountains. Male claspers from these two areas are clearly distinct, but the change is gradual across the populations connecting them; preliminary analyses of geographic song variation suggest a similar pattern, indicating a possible multicharacter cline (Strazanac, 1996, unpublished data). Our sampling is far too limited to address this issue directly, but, as discussed above, we did identify a remarkable level of genetic variation on Hawaii. Thus, it appears likely that more than one *Banza* species occurs on Hawaii. Our genetic data suggest only that "*nitida*" from the Kohala Mountains may represent a distinct species, but clinal variation around the island perimeter, as suggested by Strazanac, may occur as well.

The work presented here establishes a solid foundation for future investigations of this fascinating group. Future sampling and analysis can now be focused on addressing

highlighted questions, such as whether substitution rates for nuclear genes and mtDNA are consistently high, what the relationships are among *Banza* on Hawaii and Maui, and the nature of historical or contemporary gene flow between *B. parvula* and *B. unica* on Oahu. These studies, which will need to involve both genetic data (allozymes and other nuclear markers as well as mtDNA) and breeding and behavioral data, hold great promise for broadening our understanding of speciation in the Hawaiian Archipelago.

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Appendix A

Location data for *Banza* individuals sampled and GenBank accession numbers for sequences used in our analyses

Taxon	Island	Location	Latitude	Longitude	COI Accession No.	Cytochrome *b* Accession No.
B. nihoa (A)	Nihoa	Miller Peak	23°03′44″N	161°55′34″W	DQ649491	DQ649515
B. nihoa (B)	Nihoa	Miller Peak	23°03′44″N	161°55′34″W	DQ649492	DQ649516
B. kauaiensis (A)[a]	Kauai	Alexander Dam	21°58′30″N	159°27′58″W	DQ649483	DQ649507
B. kauaiensis (B)[b]	Kauai	Kokee, Pihea Trail	22°09′14″N	159°37′30″W	DQ649484	DQ649508
B. unica (A)[c]	Oahu	Mount Tantalus	21°20′14″N	157°49′04″W	DQ649501	DQ649525
B. unica (B)[d]	Oahu	Palikea Trail	21°24′59″N	158°06′13″W	DQ649502	DQ649526
B. parvula (A)	Oahu	Puu Kaua	21°26′41″N	158°06′05″W	DQ649497	DQ649521
B. parvula (B)	Oahu	Peacock Flats	21°32′57″N	158°11′13″W	DQ649498	DQ649522
B. molokaiensis (A)	Molokai	Puu Kole Kole	21°06′35″N	156°54′10″W	DQ649487	DQ649511
B. molokaiensis (B)	Molokai	Puu Kole Kole	21°06′35″N	156°54′10″W	DQ649488	DQ649512
B. deplanata (A)	Lanai	Lanaihale	20°48′53″N	156°52′33″W	DQ649481	DQ649505
B. deplanata (B)	Lanai	Lanaihale	20°48′53″N	156°52′14″W	DQ649482	DQ649506
B. brunnea (A)	Maui (West)	Kaulalewelewe	20°56′14″N	156°37′10″W	DQ649479	DQ649503
B. brunnea (B)	Maui (West)	Lihau	20°51′18″N	156°36′12″W	DQ649480	DQ649504
B. mauiensis (A)	Maui (West)	Hanaula	20°50′42″N	156°33′26″W	DQ649485	DQ649509
B. mauiensis (B)	Maui (West)	Hanaula	20°50′42″N	156°33′26″W	DQ649486	DQ649510
B. "*pilimauiensis*" (A)	Maui (East)	Waikamoi	20°49′04″N	156°13′49″W	DQ649499	DQ649523
B. "*pilimauiensis*" (B)	Maui (East)	Waikamoi	20°49′04″N	156°13′49″W	DQ649500	DQ649524
B. nitida (A)	Hawaii	Kealakekua	19°30′32″N	155°51′46″W	DQ649493	DQ649517
B. nitida (B)	Hawaii	Stainback	19°34′14″N	155°11′19″W	DQ649495	DQ649519
B. nitida (C)[e]	Hawaii	Puu Iki	20°07′45″N	155°46′09″W	DQ649494	DQ649518

[a] Form "*kauaiensis*".
[b] Form "*affinis*".
[c] Ko'olau Mountains.
[d] Wai'anae Mountains.
[e] Kohala Mountain.

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