

Short Communication

Mitochondrial phylogeny of the endemic Hawaiian craneflies (Diptera, Limoniidae, *Dicranomyia*): Implications for biogeography and species formation

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1. Introduction

Situated in the middle of the Pacific Ocean, over 3000 km from the nearest continental land mass, the Hawaiian Archipelago is the most remote island group on earth. The Hawaiian Islands are often referred to as a “conveyor belt” archipelago (Carson and Clague, 1995); as the Pacific plate moves to the northwest over a stationary hotspot, islands are formed, carried with the plate, and gradually subside at a more-or-less constant rate (Price and Clague, 2002; Ziegler, 2002). This hotspot has been active for over 40 million years (Carson and Clague, 1995) and has resulted in a chain of successively older islands, seamounts, and atolls stretching northwest across the Pacific. Currently, there are eight “high islands” in the southeast of the chain that, at over 1000 m in elevation, are able to support a diverse flora and fauna. The youngest island, dated at less than 600,000 years old, is the Big Island of Hawai’i. Kaua’i, at about 5 million years old, is the oldest high island (Price and Clague, 2002).

Extreme isolation, coupled with the unique geology and environmental conditions of the islands, has made Hawai’i a “natural laboratory” where the effects of evolution are readily observable (Kaneshiro, 1995). Over 850 endemic flowering plant (Wagner et al., 1990) and 4000 endemic insect species (Liebherr, 2001) are each thought to have been derived from about 200 colonization events. Colonist species can rapidly fill open ecological niches and diversify *in situ*, resulting in an amazing array of endemic fauna and flora. Newly emerging islands are then colonized from

older source populations. These new, allopatric populations can, over time, increase biodiversity and lead to a high number of single island endemic species.

The origin and subsequent diversification of the Hawaiian biota has been the subject of a great deal of research, including studies on insects (Bonacum et al., 2005; Mendelson and Shaw, 2005; Shapiro et al., 2007), birds (honeycreepers, see Jarvi et al., 2004), spiders (Gillespie et al., 1994) and plants (silversword alliance, see Baldwin and Robichaux, 1995). Although each species has a unique evolutionary history, comparisons across studies can elucidate common evolutionary patterns (Wagner and Funk, 1995; Price and Clague, 2002). One such common pattern is the progression rule (Hennig, 1966; Wiley, 1981; Wagner and Funk, 1995), where the most basal species in a phylogeny are found on the oldest islands and the more derived species occupy younger islands. Although this pattern is observed in many groups of species (e.g., Bonacum et al., 2005; Wagner and Funk, 1995), there are also many exceptions to this rule where a different form of dispersal and speciation seems to be at work (Lowrey, 1995). Such examples might include diversification within islands, back migration from younger to older islands, or a stochastic process that does not result in any distinct pattern.

The Diptera represent over 10% of the known endemic insect fauna of the Hawaiian Islands. Three large families, Drosophilidae, Dolichopodidae, and Muscidae, each have hundreds of known species (Eldredge and Evenhuis, 2003; Nishida, 2002; O’Grady, 2002). In addition to these large groups, several other families of Diptera have undergone smaller radiations of 10–50 species (Nishida, 2002). By comparing phylogenetic and biogeographic patterns between larger and smaller families, we hope to gain insight into how diversification may have taken place in these

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groups. Examining factors such as age of colonization and rate of speciation may yield a better understanding of the evolutionary dynamics involved in the formation of island radiations.

The endemic Hawaiian *Dicranomyia* (Diptera: Limoniidae) are an excellent point of comparison for the patterns observed in other groups of Hawaiian Insects. These crane-flies are long-legged, slender-bodied species, living in a variety of aquatic or semi-aquatic habitats (Hardy, 1960). In his taxonomic revision, Hardy (1960) listed a total of 10 species, along with 2 subspecies. Byers (1982, 1985, 1994) added three flightless species to this list, bringing the total number of known Hawaiian species to 13. Unlike the Hawaiian *Drosophila*, in which over 90% of species are single island endemics, 75% of *Dicranomyia* species are found on multiple islands. Only three crane-fly species, all of which are flightless, are restricted to single islands. The remaining taxa are found on more than one island and seven are found on all the high islands. Although very little research has been done on the group, the data amassed thus far points to a dispersal pattern that does not follow the progression rule. In the current study we generate a molecular phylogeny for the endemic crane-flies using a suite of four mitochondrial loci and infer the biogeographic history of the group using parsimony and likelihood methods to reconstruct patterns of island colonization.

2. Methods

2.1. Taxon sampling

All material was obtained from general sweeping of vegetation, seeps and streams. Samples were preserved in 95% ethanol (ETOH) and transported to UC Berkeley for identification and subsequent molecular work. Hardy's (1960) key and descriptions were used to identify the Hawaiian *Dicranomyia*. Material from French Polynesia was identified using a number of references (Alexander, 1914, 1921, 1932, 1933, 1935, 1947; Brunetti, 1912; Edwards, 1927, 1928). Wings and genitalia were mounted and preserved from all specimens sacrificed for DNA. When possible, a conspecific series of specimens collected at the same site as those extracted was also preserved in 95% ETOH. Outgroups were selected from species thought to be closely related to the Hawaiian species. These include a congener from French Polynesia, *D. tahitiensis*, and representatives of two genera, *Geranomyia* and *Libnotes*, which were once considered subgenera of *Dicranomyia* (Hardy, 1960) and are currently placed in the subfamily Limoniinae (Oosterbroek, 2007).

Appendix 1 lists the species and authority, collection information and O'Grady Lab collection number of all taxa sampled in the current study. We sampled a total of 8 of the 13 described species of Hawaiian *Dicranomyia*. Three of the remaining taxa, all described by Byers (1982, 1985, 1994), are flightless and known only from a few specimens collected over the past 100 years. It was

not possible to include these taxa. All Hawaiian *Dicranomyia* included in this study are known from multiple islands and, when possible, representatives from each island within a given species' known range were included (Appendix 1). Voucher material has been deposited in the Bernice P. Bishop Museum (Honolulu, HI) and the Essig Museum of Entomology at UC Berkeley.

2.2. Extraction, amplification, and sequencing of DNA

All samples were macerated in a 1.7 mL microcentrifuge tube with a micropestle, and nucleic acids were extracted using the Qiagen DNeasy[®] (Qiagen Inc.) tissue kit following the manufacturer's protocol. We used four mitochondrial loci to estimate phylogenetic relationships within this group. Primer names correspond to location in the *Drosophila yakuba* mitochondrial genome (Clary and Wolstenholme, 1985). A 529 bp fragment of the ND2 gene was amplified using primers 192 and 732 (Bonacum et al., 2001). A ~1600 bp fragment containing portions of the COI and COII genes was amplified using primers 2183, 2460, 3037, 3041, and 3771 (Bonacum et al., 2001). A 534 bp portion of the 16S locus was amplified with primers 16SF and 16SR (DeSalle, 1992). PCR was performed under the following conditions: initial denaturing step at 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, then finally held at 20 °C until removal from the machine. Samples were purified with ExoSapIt (Amersham) and sent to the UC Berkeley DNA Sequencing Center.

2.3. Sequence alignment and phylogenetic analysis

Sequences were edited in Sequencher 4.0 (Gene Codes Corp.) and exported as NEXUS formatted files (Maddison et al., 1997) for alignment and analyses. The alignment of the three protein coding loci in this study, ND2, COI, and COII, was trivial and done by eye. The 16S region required only a few small gaps throughout its length. One taxon, *Dicranomyia* sp. 200984, had a ~20 bp TA repeat in the 3' region of this sequence.

Maximum parsimony analyses were performed in PAUP* 4.0 (Swofford, 2002) with a heuristic search algorithm (addition sequence = random; number of replicates = 1000; swapping = TBR). Loci were analyzed both individually (Fig. 1) and in a single concatenated data matrix (Fig. 2). Support was assessed using 1000 bootstrap replicates (BP; Felsenstein, 1988) with the same settings as above. Table 1 shows the size, number of parsimony informative characters, number and length of shortest parsimony trees of each data set analyzed. Individual gene analyses included all taxa for which a given locus was available.

Modeltest, version 3.8 (Posada and Crandall, 1998), was used to select an appropriate model of evolution under the BIC (Table 2). This model was then implemented in the maximum likelihood (PAUP* 4.0; Swofford, 2002) and

Bayesian (MrBayes 3.1.1; Huelsenbeck and Ronquist, 2001) analyses, in order to infer phylogenetic relationships. Bayesian analyses using the appropriate model (Table 2) were implemented as follows: two sets of four chains were allowed to run simultaneously for 1,000,000 generations. Each set was sampled every 100 generations with a burnin of 10%. Degree of support at each node was measured with posterior probabilities (PP). Convergence of chains was assessed by examining the average standard deviations of split frequencies and potential scale reduction factor (PSRF) values (Huelsenbeck et al., 2001).

Combined analyses explored the differences between sampling fewer taxa with no missing data and more inclusive taxon sampling with a higher percentage of missing data. All 17 taxa in the “all 4 loci” analysis (Fig. 2c) were complete for the ~2.6 kb of sequence generated in this study. The “3 of 4 loci” and “2 of 4 loci” analyses (Fig. 2a and b) had broader taxon sampling (32 and 38 taxa, respectively) but also contained a modest amount of missing data.

Biogeographic scenarios were explored by estimating ranges of putative ancestors based on distribution of extant

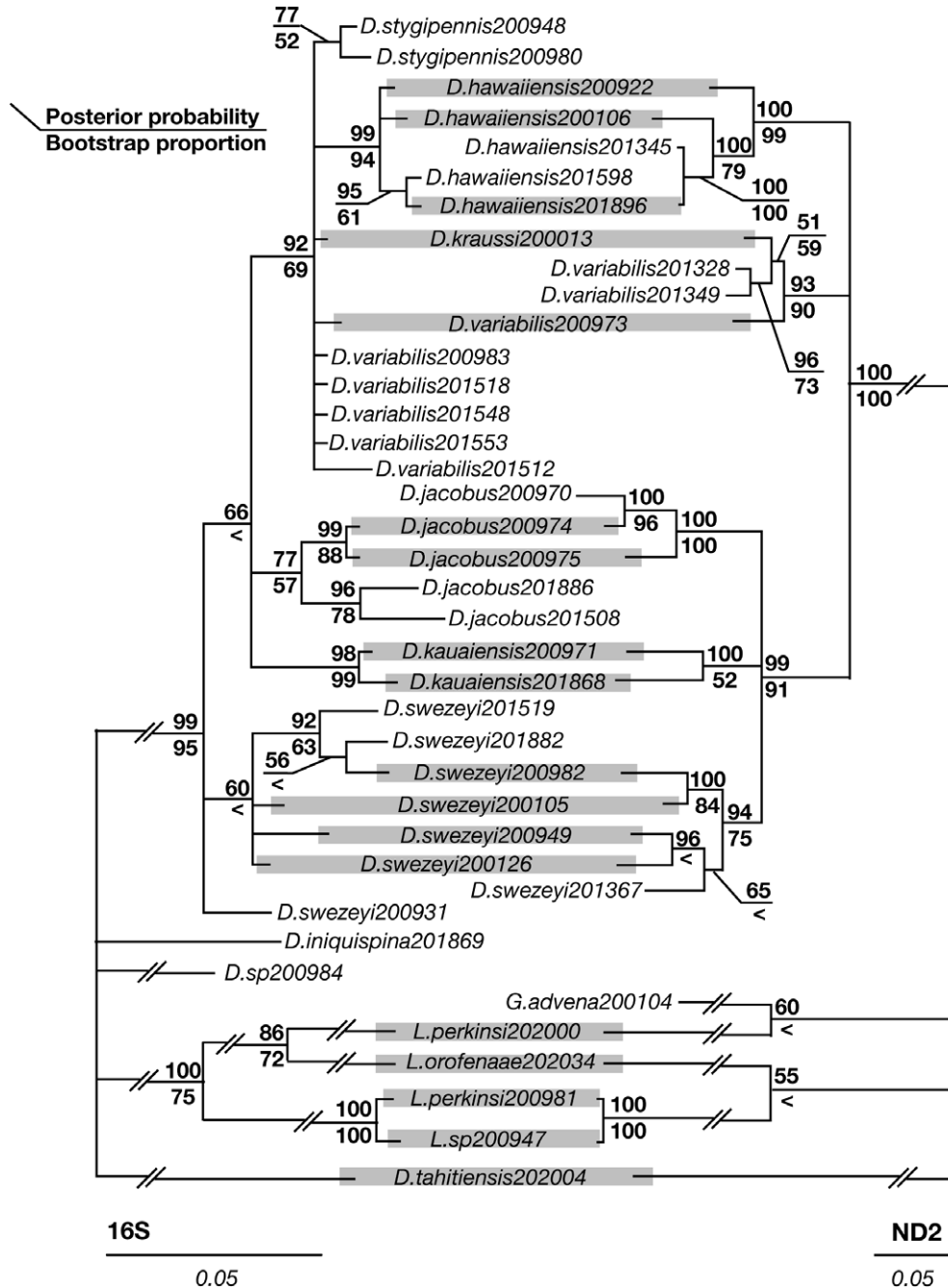


Fig. 1. Individual analyses of the (a) 16S, (b) ND2, (c) COI and (d) COII loci. Branch lengths are drawn proportional to change, except where hatch marks are present. Posterior probability values are above the nodes, bootstrap proportions greater than 50% are below. Taxa shaded in gray are present in both analyses.

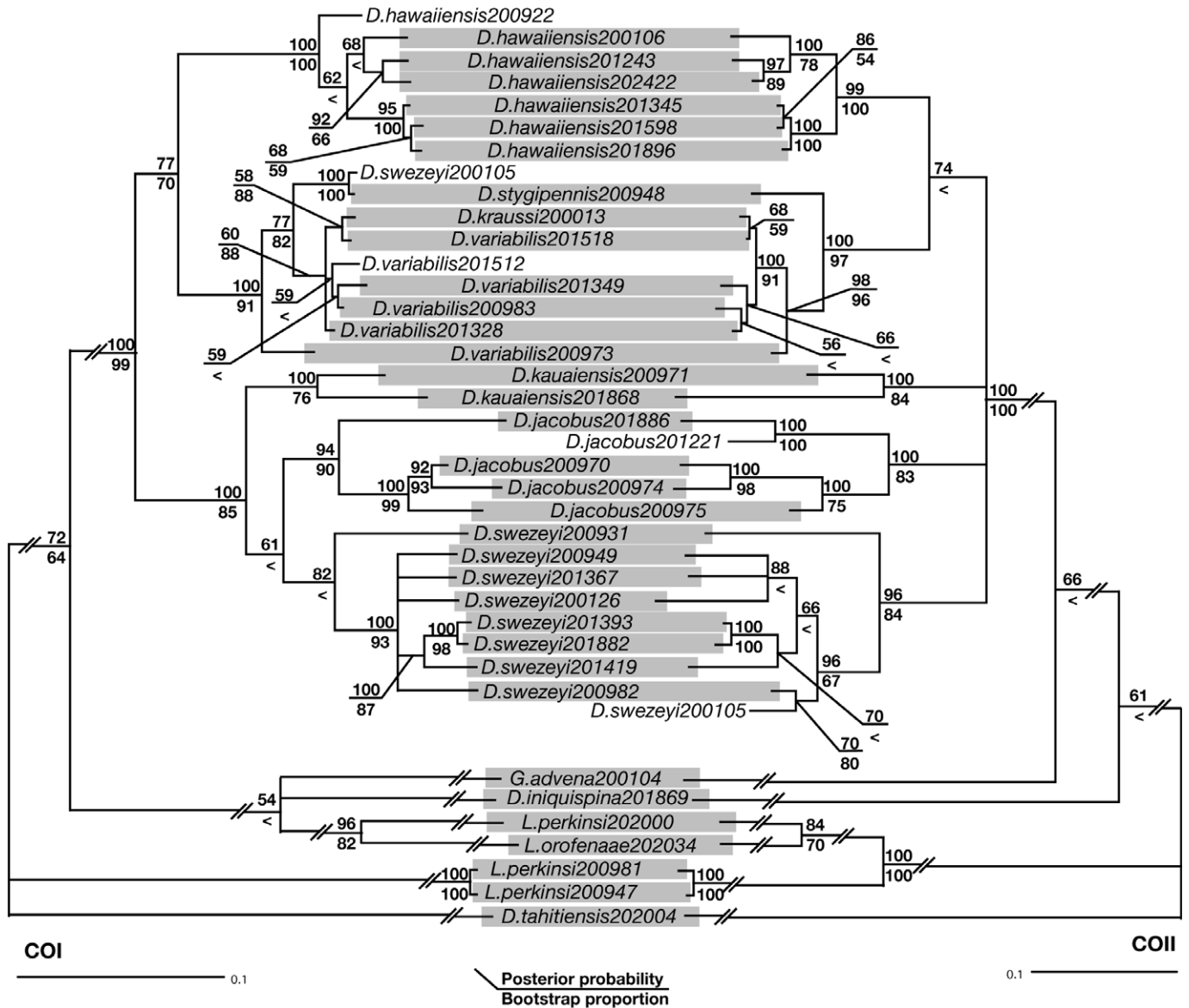


Fig. 1 (continued)

taxa. Both parsimony and likelihood based methods were used to analyze the data in Mesquite v1.06 (Maddison and Maddison, 2007). Current island distribution was coded as a five-state (Kaua'i, O'ahu, Moloka'i, Maui, Hawai'i), unordered character. No weighting was assigned to the parsimony step matrix. For likelihood analysis, the Markov k-state 1 model was used with parameters estimated by Mesquite based on the data matrix (Lewis, 2001).

3. Results and discussion

3.1. Individual analyses

Individual analyses on all sequences generated for each of the four partitions, 16S, COI, COII and ND2 were performed using maximum parsimony, maximum likelihood, and Bayesian analyses. Trees inferred by the three method-

ologies were not topologically different from one another (data not shown). Fig. 1a–d shows the results of Bayesian analyses for each individual gene, with posterior probabilities and maximum parsimony bootstrap proportions at each node. Comparisons between loci show little significant difference in topology of well-supported nodes, only variations in levels of support and resolution between trees. All analyses strongly support the monophyly of all endemic Hawaiian *Dicranomyia*, with the exception of *D. iniquispina*. This species is genetically distinct from the other taxa and may represent a separate colonization event, although bootstrap support for this assertion is not strong.

Several species (*D. hawaiiensis*, *D. jacobus*, *D. kauaiensis*, *D. stygipennis*) were each supported as monophyletic in individual analyses including two or more populations (Fig. 1a–d), although populations from a given island were not always supported as monophyletic. Other taxa, such as

D. variabilis, were monophyletic in some (Fig. 1b), but not all (Fig. 1a, c and d), analyses. *Dicranomyia kraussi*, a species that is morphologically very similar to *D. variabilis*, renders the latter species paraphyletic in most analyses. One taxon, *D. swezeyi*, was monophyletic in some (ND2 and COI) but not all (COII and 16S) analyses, in spite of the fact that this species is morphologically distinct from the other Hawaiian *Dicranomyia*. This species seems to consist of a number of separate lineages, in spite of the fact that the male genitalic and wing characters that define this taxon are invariant between populations. It may be possible that 2–3 cryptic species are present within what is currently defined as *D. swezeyi* or that hybridization has played a role in the history of this taxon.

Relationships between species or groups of species were not well supported in most of the individual analyses. In fact, basal relationships in three of the four individual phylogenies were not resolved. The COI gene did show modest

support for two clades, one containing *D. swezeyi* (except for isolate 200105), *D. jacobus*, and *D. kauaiensis* and another consisting of *D. hawaiiensis*, *D. stygipennis*, *D. kraussi*, and *D. variabilis*.

3.2. Combined analyses

Combined analyses using parsimony (data not shown), likelihood (data not shown) and Bayesian (Fig. 2a–c) methods were carried out on a number of taxon inclusion sets; those species sampled for all four loci, three of four loci, and two of four loci (i.e., the largest set of taxa for which any two or more loci were sequenced). There is little difference in the relationships supported by any of the three analytical methods or the three taxon sampling schemes. We have also performed all pairwise and three-way combinations of genes (Supplementary data) and the results are concordant with Fig. 2. There is no change in relationship

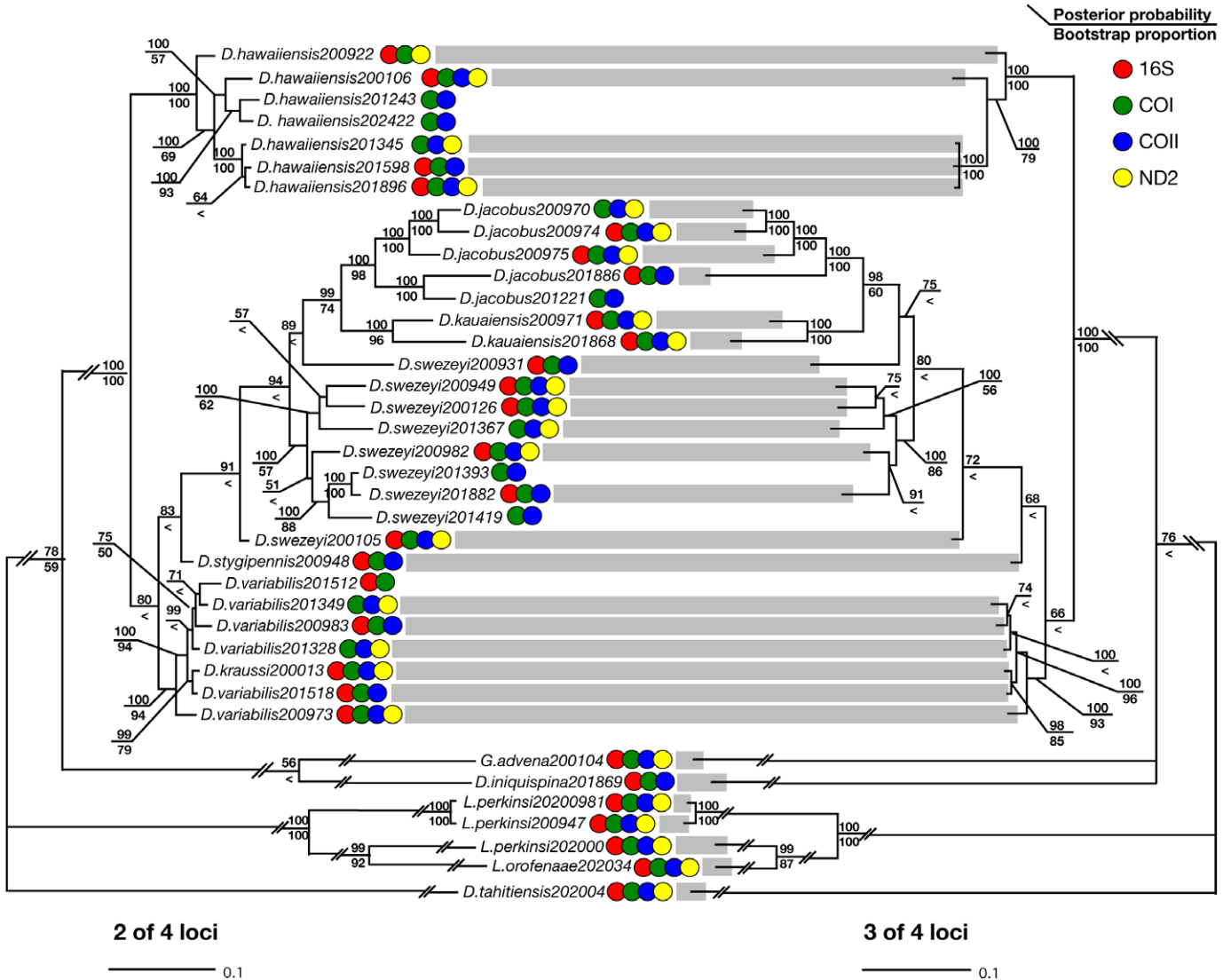


Fig. 2. Combined analyses of the (a) 2 of 4, (b) 3 of 4 and (c) all four taxaset. Branch lengths are drawn proportional to change, except where hatch marks are present. Posterior probability values are above the nodes, bootstrap proportions greater than 50% are below. Taxa shaded in gray are present in both analyses.

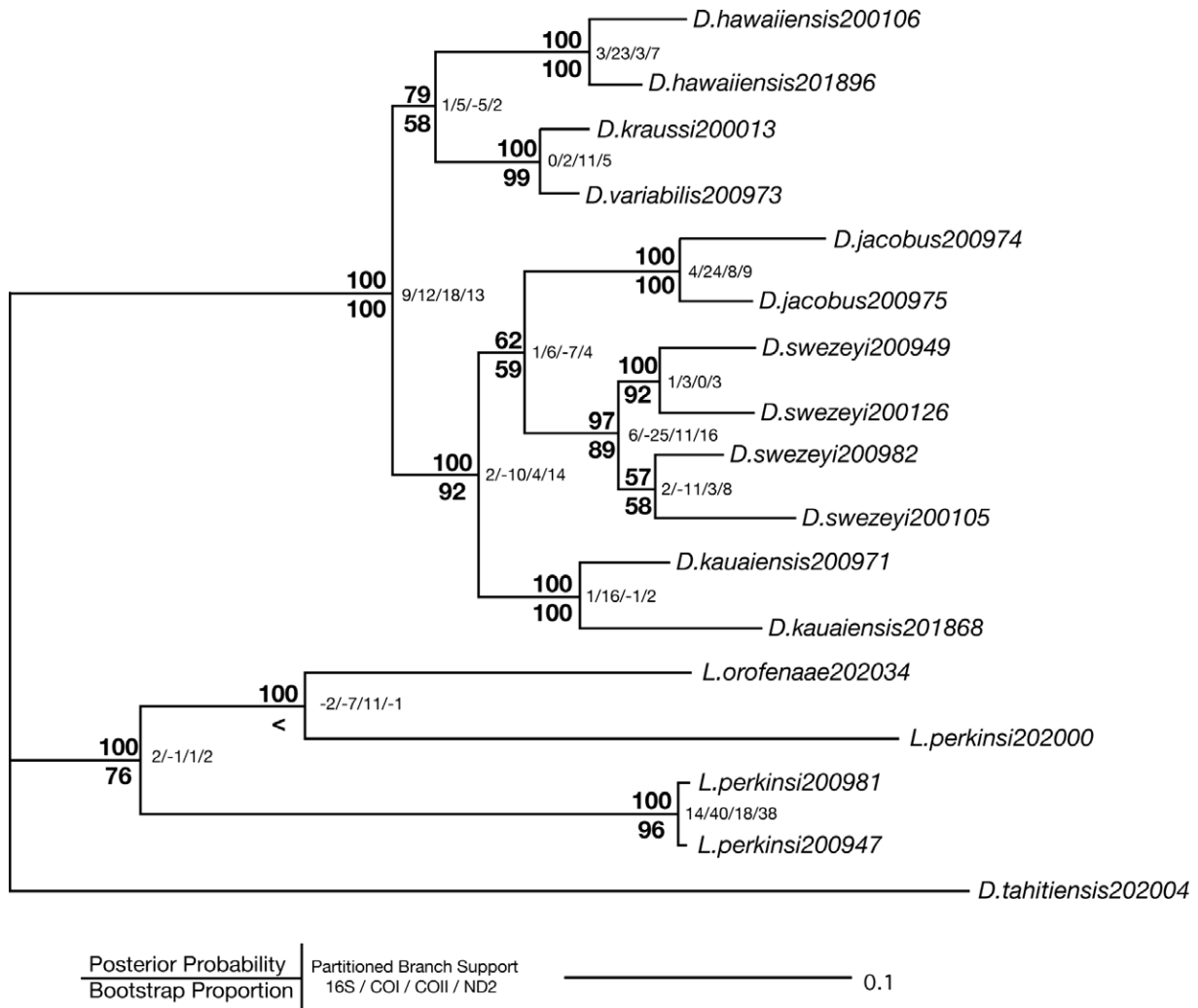


Fig. 2 (continued)

Table 1
 Summary of individual and combined maximum parsimony analyses

Partition	# Chars (PICs) ^a	# Taxa ^b	# Trees ^c	# Islands ^d	Treelength ^e	CI ^f	RI ^g
ND2	532 (201)	24	1	1	695	0.56	0.63
COI	824 (295)	38	18	1	1224	0.40	0.63
COII	767 (229)	36	10	1	1044	0.44	0.62
16S	534 (76)	33	6	1	234	0.66	0.74
All 4	2657 (682)	17	2	1	2189	0.56	0.54
3 of 4	2657 (758)	32	2	1	3088	0.46	0.56
2 of 4	2657 (769)	38	17	2	3258	0.45	0.61

^a Number of characters (number of parsimony informative characters).

^b Number of taxa analyzed.

^c Number of most parsimonious trees found.

^d Number of islands of most parsimonious trees discovered.

^e Treelength of most parsimonious trees.

^f Consistency index.

^g Retention index.

as number of taxa, and percentage of missing data, increases (Fig. 2a–c). Furthermore, aside from increased support for basal nodes in analyses with more characters, there was little difference in topology between the individual and combined analyses.

The analysis of all taxa with at least two of the four loci determined is shown in Fig. 2a. With the exception of *D. iniquispina*, all Hawaiian *Dicranomyia* are strongly supported as monophyletic (PP, BP = 100) and are the result of a single colonization event. Although *D. iniquispina*

Table 2
Summary of initial model parameters used in maximum likelihood and Bayesian analyses

Partition	Model	−lnL	G ^a	I ^b	Base frequencies		Rate matrix		
ND2	HKY+I+G	4271.4116	0.7169	0.3193	A = 0.3740 G = 0.0549	C = 0.0943 T = 0.4767	Ti/Tv Ratio = 2.4169		
COI	HKY+I+G	7200.1206	0.6967	0.5208	A = 0.3794 G = 0.0579	C = 0.1045 T = 0.4581	Ti/Tv Ratio = 4.7758		
COII	GTR+I+G	6729.0889	0.5713	0.4155	A = 0.3939 G = 0.0699	C = 0.0999 T = 0.4363	A–C = 3.5673 C–G = 11.2384	A–G = 16.9792 C–T = 35.9973	A–T = 2.1139 G–T = 1.0000
16S	GTR+I+G	2335.3525	0.742	0.4438	A = 0.3854 G = 0.0903	C = 0.131 T = 0.3933	A–C = 0.4743 C–G = 0.0000	A–G = 8.6377 C–T = 15.4949	A–T = 16.7939 G–T = 1.0000
All 4	GTR+I+G	13541.3779	0.7748	0.4849	A = 0.3517 G = 0.1015	C = 0.1089 T = 0.4379	A–C = 2.8825 C–G = 3.8386	A–G = 15.4518 C–T = 57.7085	A–T = 8.552 G–T = 1.0000
3 of 4	GTR+I+G	17657.6758	0.7374	0.4904	A = 0.3607 G = 0.0863	C = 0.1094 T = 0.4436	A–C = 2.7301 C–G = 3.9139	A–G = 17.0522 C–T = 35.1806	A–T = 4.7407 G–T = 1.000
2 of 4	GTR+I+G	18768.2129	0.7202	0.4930	A = 0.3650 G = 0.0805	C = 0.1099 T = 0.4447	A–C = 2.3432 C–G = 3.3651	A–G = 16.9366 C–T = 30.1594	A–T = 3.7054 G–T = 1.0000

^a Gamma shape parameter.

^b Proportion of invariant sites.

may be the result of an independent colonization, there is little statistical support at basal nodes in this phylogeny. *Dicranomyia jacobus* and *D. kauaiensis* are both strongly supported as monophyletic (PP = 100, BP = 98 and PP = 100, BP = 96, respectively) and are sister to one another (PP = 99, BP = 74). While *D. swezeyi* is monophyletic in the smaller combined analysis (Fig. 2c), analyses that sampled more broadly within this species (Fig. 2a and b) show populations forming a paraphyletic grade basal to the *jacobus-kauaiensis* sister pair. Posterior probability values supporting this grade are high (89, 100, 91) but bootstrap proportions (<50, 57, <50) suggest that the exact relationships within *D. swezeyi* populations are not well supported and will require additional characters before they can be resolved. A single isolate of *D. stygipennis* is basal to the *D. swezeyi* grade. A clade (PP = 100, BP = 94) consisting of *D. kraussi* and *D. variabilis* is basal to *D. stygipennis*. Morphologically, *D. kraussi* and *D. variabilis* are very close and may in fact overlap in the single character that is able to diagnose these taxa, the shape of the ventromesal lobe of the epandrium. Relationships within this group are not well supported (BPs < 80). *Dicranomyia hawaiiensis* is strongly supported as monophyletic (PP, BP = 100) and sister to the remaining Hawaiian taxa.

3.3. Biogeographic patterns

The current study suggests that the endemic Hawaiian *Dicranomyia* may be the result of at least two colonization events, one by the bulk of species analyzed here and another by *D. iniquispina* (Fig. 2a–c), although more support at basal nodes will be needed to address the possibility of a second colonization. The fact that the genus *Dicranomyia* is widespread across the Pacific (Evenhuis, 2007) and that the morphological concept for this genus has undergone much modification (Hardy, 1960; Oosterbroek, 2007) and continues to be difficult to resolve, suggest

that more than a single colonization might be plausible. Future research that samples *Dicranomyia* from a number of Pacific Islands including Fiji, French Polynesia, and Samoa, will be needed to determine whether *D. iniquispina* is sister to the Hawaiian taxa or is actually the result of a second colonization.

Biogeographic patterns within the Hawaiian *Dicranomyia* are complex and do not strictly adhere to the progression rule or any other simple pattern of dispersal observed in a number of endemic Hawaiian plant and insect lineages (Bonacum et al., 2005; Wagner and Funk, 1995). In fact, maximum likelihood methods reconstruct the ancestral state at each node as equally probable between the five islands mapped (data not shown). While parsimony is more conclusive about some of the nodes in this phylogeny, the basal node is still unresolved between the oldest island, Kaua'i, and the youngest, Hawai'i (Fig. 3). Additional taxon sampling (e.g., of rare flightless species) may help resolve the issue of ancestral state reconstructions, but is not likely to alter the violation of the progression rule that these data support. Within some species (*D. hawaiiensis*, *D. variabilis*) there is a basal population on Kaua'i with a complex pattern of subsequent radiation, back colonization, and island skipping. Other species, such as *D. swezeyi*, seem to have originated on a younger island and subsequently colonized older islands, a pattern also observed in the native Hawaiian *Hylaeus* bees (Magnacca and Danforth, 2006).

Although craneflies are not generally considered strong fliers, it is possible that the complex patterns observed are due to the fact that *Dicranomyia* are likely to be blown from one island to another. In addition to being present in deep rainforest habitats, several populations were collected high on volcanoes (Mauna Loa), on windswept ridges (Kaua'i, Moloka'i) and in low elevation, disturbed habitats (Kalopa). Populations present in such localities may be highly susceptible to updrafts and could be transported from one island to another.

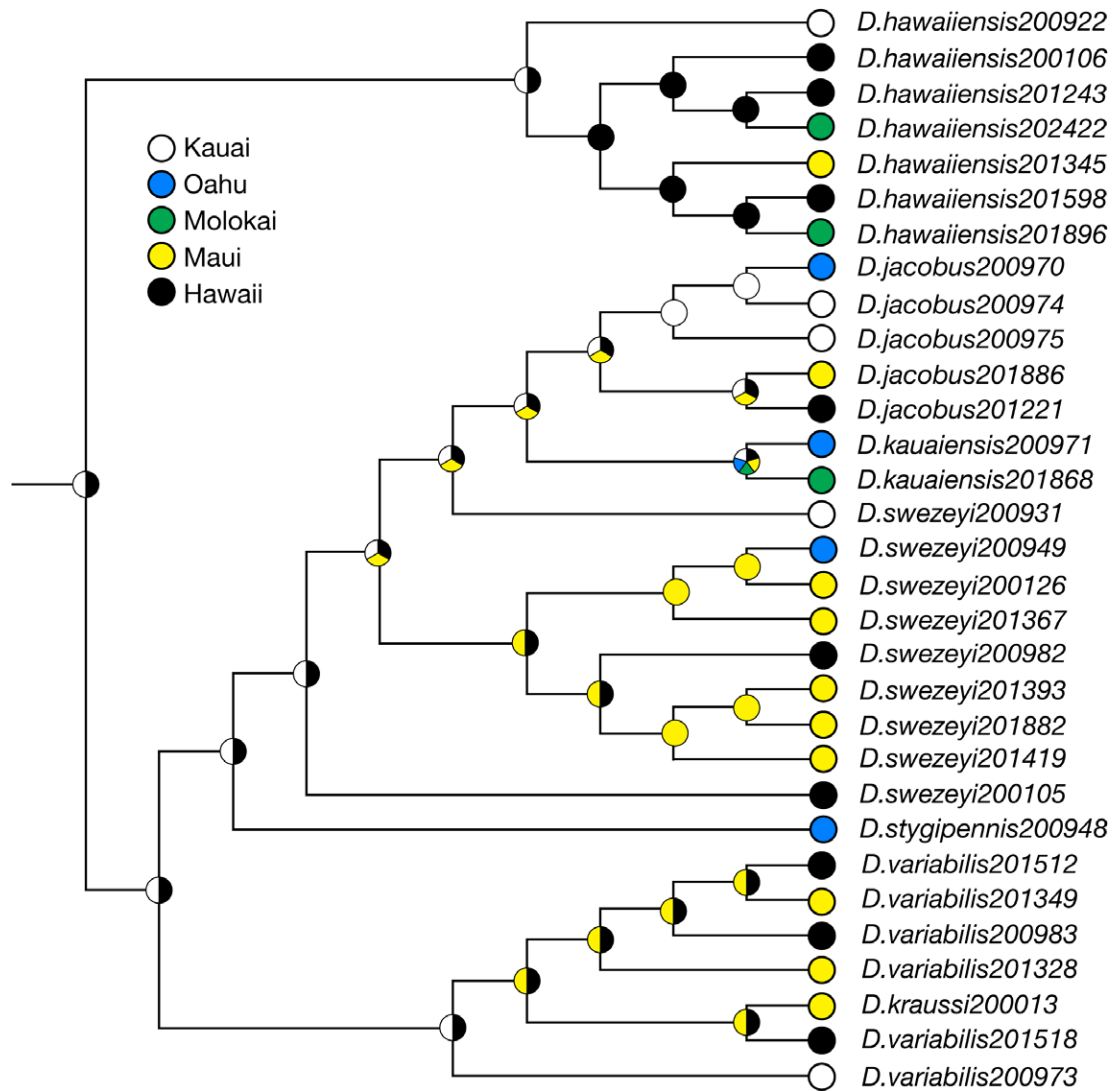


Fig. 3. Biogeographic analysis of the 2 of 4 analysis (Fig. 2A), with parsimony reconstructions shown at each node.

4. Conclusions

The present study is a preliminary investigation into the phylogenetic relationships and biogeographic patterns in the endemic Hawaiian *Dicranomyia*. The complex patterns of dispersal within this group stand in stark contrast to the simple patterns seen in other Hawaiian groups (e.g., Hawaiian *Drosophila*, Bonacum et al., 2005). The genus *Dicranomyia* is found on islands throughout the Pacific where it seems to have diversified extensively. This group will be an interesting model system for future biogeographic studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2007.12.021](https://doi.org/10.1016/j.ympev.2007.12.021).

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