CHAPTER 3

Host-plant shifts and interisland dispersal in the evolution of Hawaiian *Cydia* Hübner (Lepidoptera: Tortricidae)

ABSTRACT

What drives diversification and speciation is a central topic of biodiversity research. Geographic isolation is thought to be one of the most important factors leading to new species formation, although renewed interest in ecological speciation has led to convincing evidence that niche partitioning and symbiotic relationships can play an equally important role in speciation in some biological systems. To evaluate the relative importance of geography and ecology in diversification I mapping the distribution and host-plant affinities of 14 species of Hawaiian *Cydia* moths on a molecular phylogeny constructed from nuclear and mitochondrial genes. The genus *Cydia* is represented by a monophyletic clade of at least 21 endemic species distributed throughout the Hawaiian Islands and feed on endemic plants in the family Fabaceae. I show that diversification of this genus in Hawaii is associated with host-plant shifts followed by dispersal to similar niches on other islands. I also show that *Cydia pseudomalesana* Clarke in French Polynesia is a separate colonization of the Pacific from the Hawaiian species, stemming from the Austral-Asian region. The origins of Hawaiian *Cydia* remain obscured although they appear most closely related to species in the Holarctic region.

INTRODUCTION

The relative influence of geographic isolation and ecology in the diversification of lineages has long been debated, but has been recently rekindled through renewed interest in ecological speciation (Friar et al. 2007, Egan et al. 2008, Matsubayashi et al. 2010, Peccoud and Simon 2010, Rice et al. 2011, Thibert-Plante and Hendry 2011). Host plants of phytophagous insects, in particular, can act as aggregation sites for mating, facilitate differential developmental rates and success among populations, and result in genetic differentiation through adaptive selection or genetic drift (Berlocher and Feder 2002, Thomas et al. 2003, Egan et al. 2008, Nosil et al. 2008, Matsubayashi et al. 2010, Michael and Carolyn 2010, Peccoud and Simon 2010). More often, however, differentiation among populations of phytophagous insects probably is caused by a number of interacting factors including ecological opportunity, competitive displacement, specialization, sexual selection, and reproductive success, as well as the geographic structure of populations that can minimize gene flow (Turner and Burrows 1995, Berlocher and Feder 2002, Dres and Mallet 2002, Despres and Cherif 2004, Matsubayashi and Katakura 2009, Yoder et al. 2010). As molecular tools for identifying population structure continue to expand and improve, the nexus of ecology and geography in promoting speciation are becoming better understood. However, much of our understanding is based on a limited number of model systems.

Remote island ecosystems offer a fertile laboratory for contrasting the role of ecology and geography in speciation because of their discrete geographic units and the endemic radiations often found on them. The Hawaiian Islands are home to some 6000+ native insects and spiders (Nishida 2002, Eldredge and Evenhuis 2003) that are thought to have diversified from only 233-400 independent colonizations (Zimmerman 1948, Howarth 1990). While some genera, such as *Drosophila* flies (~1000 spp., O'Grady et al. 2011) and *Hyposmocoma* moths (300+ spp., Rubinoff 2008), have diversified extraordinarily from one or few initial colonizers, others such as *Manduca* moths and *Vanessa* butterflies are known

by only a single endemic species (for review, see Howarth 1990, Roderick and Gillespie 1998). Moreover, entire families of insects that are widespread and common on continents, such as ants, hover flies, and ladybird beetles, have no native representatives in Hawaii (Nishida 2002). While contingency probably has played an important role in the establishment of new lineages, the extreme isolation of the Hawaiian Archipelago likely is responsible for the lack of many taxonomic groups, attenuation of others, and fostering of the spectacular radiations for some of those fortunate enough to reach Hawaii (Perkins 1913, Zimmerman 1948, Gillespie and Roderick 2002, Gillespie and Baldwin 2010).

Of the 20 families of moths with native species in the Hawaiian Islands, the cosmopolitan and species-rich family Tortricidae (nearly 10,000 species, Brown et al. 2005) is represented by eleven native and eight wholly non-native genera (Zimmerman 1978, Nishida 2002). The eight non-native genera, (*Acleris*, *Amorbia*, *Bactra*, *Epiphyas*, *Episimus*, *Lorita*, *Platynota*, and *Strepsicrates*), include both accidental introductions and purposefully released biological control agents (Funasaki *et al.* 1988). Of the eleven genera having native species, eight are endemic to Hawaii (*Eccoptocera*, *Macraesthetica*, *Mantua*, *Nuritamburia* [formerly *Bradleyella* (see Koçak and Kemal 2007)], *Panaphelix*, *Paraphasis*, *Pararrhaptica*, and *Spheterista*). Of the three remaining tortricid genera in Hawaii, *Cryptophlebia* includes one putatively native and one non-native species, *Crocidosema* includes three endemic species, and the genus *Cydia*, although it includes many widespread pests of legumes and conifers, is known only from 21 endemic species in Hawaii (Oboyski Chapter 2, Zimmerman 1978).

Hawaiian *Cydia* (Grapholitini) is distributed across all the high islands from shoreline to tree line and feed in the generative tissues of legumes (Fabaceae), thus forming a ubiquitous and important link in Hawaiian food webs (Table 2). Larvae of Hawaiian *Cydia* appear to be host-specific, feeding within the seeds, flowers, terminal twigs, or under bark of *Acacia koa* A. Gray, *Acacia koaia* Hillebr., *Canavalia* spp., *Sophora chrysophylla* (Salisb.) Seem., *Strongylodon ruber* Vogel, and *Vicia menziesii* Spreng. (Swezey 1954, Zimmerman 1978), consuming up to 70% of the seed crop in the case of *Sophora* (Swezey in Zimmerman 1978). Larvae also provide a rich protein source for rare endemic Hawaiian birds such as the akiapola'au (TK Pratt and PT Oboyski unpublished data) and palila (Banko et al. 2002b), and share a suite of parasitoid wasps with agricultural pests and other native moth species (Zimmerman 1978, Brenner et al. 2002, Oboyski et al. 2004). Despite their ecological importance, however, the evolutionary history of Hawaiian *Cydia* is largely unknown.

In addition to improving our taxonomic and ecological understanding of this group, resolution of the evolutionary history of Hawaiian *Cydia* would provide an opportunity to test hypotheses regarding modes of speciation within lineages of vagile, monophagous insects. Hawaii has long been recognized as an ideal natural laboratory for studying the processes of evolution due to the extreme isolation of the archipelago and the known ages of the linearly arranged high islands (Price and Clague 2002). Given the chronological arrangement of the islands, Funk and Wagner (1995) formalized a "progression rule" of diversification whereby lineages established on older islands gave rise to new species on younger islands. In contrast, Ehrlich and Raven (1964) suggested that a shift to new host plants could lead to speciation in tightly coupled plant-herbivore interactions. The legume

hosts on which Hawaiian *Cydia* feed represent diverse lineages within the Fabaceae, which typically produce a diverse array of secondary compounds including canavanine, β-cyanoalinine, and quinolizidine alkaloids thought to deter many herbivores (Bell 1972, Banko et al. 2002a). Therefore a shift to a new host plant accompanied by adaptations to detoxify or sequester host toxins could represent a "key innovation" that leads to diversification (Berenbaum et al. 1996).

Although Hawaii is rich with endemic, host-specific, insect herbivores, surprisingly few studies have sought to compare the relative influence of biogeography and host-plant use on speciation in a phylogenetic framework. Here I present a molecular phylogeny of Hawaiian *Cydia* to test hypotheses regarding the evolutionary history of this genus in the Hawaiian Islands. More specifically, using DNA sequence data from Hawaiian *Cydia* and their non-Hawaiian relatives I address the following questions: 1) Are Hawaiian *Cydia* a monophyletic lineage? 2) What are the likely origins of Hawaiian *Cydia*? 3) What are the biogeographic patterns of species groups relative to present-day distributions and host-plant usage? 4) What are the likely modes of diversification within Hawaiian *Cydia*?

METHODS

Taxon sampling

Specimens used in molecular analyses were collected for that purpose in the Hawaiian Islands (Figure 1) between 2002 and 2006. Additional outgroup specimens were collected in California, Mississippi, Portugal, Reunion Island, Japan, Micronesia, and French Polynesia by the author or colleagues (Table 1). Adult moths were collected using 15W ultraviolet lights powered by 12v DC batteries in targeted habitats in Hawaii (i.e. habitats supporting the known host plants of Hawaiian *Cydia*) and opportunistically elsewhere. Some adult specimens were collected using an insect net by sweeping vegetation or aerial capture of day-flying individuals. Moths were dispatched at the time of capture using potassium cyanide or frozen alive within a few hours after capture. Shortly thereafter, the middle and hind legs on one side of each specimen were pulled free from the body and placed in 95% ethyl alcohol for subsequent analysis. Moths were then pinned for identification and morphological analysis (e.g. genitalia dissections to confirm identification).

Larvae were also collected for molecular analyses and to assess host plant affinities. Known and suspected host plants were inspected for evidence of larvae boring into seeds, flowers, and twigs, and under bark. Host plant material was placed in 240 ml clear plastic containers fitted with screen lids and checked periodically for emergence of adult moths or parasitoid wasps. Some larvae were sacrificed before pupation and preserved in 95% ethyl alcohol. Both sacrificed larvae and emerged adults from host plants were used for DNA sequence analysis. Although in some cases several individuals from each location were sequenced from either captured adults or reared larvae, specimens with identical sequences from the same island were not included in the following analyses.

DNA sequencing

Total genomic DNA was extracted from one or two legs (adult moths) or a section of abdominal muscle (larvae) using a DNEasy® tissue kit (Qiagen Corporation) following the manufacturer's protocol for animal tissue. Fragments of the mitochondrial (mtDNA) gene

regions cytochrome c oxidase subunit I (COI) and subunit II (COII), the nuclear ribosomal RNA (rRNA) gene region 28S (domain 'A'), and the nuclear gene (nDNA) regions elongation factor 1 alpha (EF1α) and wingless (WG) (Table 3) were amplified using the polymerase chain reaction (PCR) (Table 4). The PCR thermal profile consisted of 2 minutes at 95 °C; 35 cycles of 30 seconds at 95 °C, 45 seconds at X °C (where annealing temperature X = 50, 63, 64 °C for the mtDNA & 28S genes, EF1α, and WG, respectively), and 90 seconds at 72 °C; and an extension cycle of 10 minutes at 72 °C. PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, Ohio) following manufacturer's specifications but at one-tenth the recommended concentration. Cycle sequencing of purified PCR products was done in both forward and reverse directions for each specimen using BigDye® v3.1 sequencing kit (ABI) following the manufacturer's protocols and subsequently cleaned by EtOH / EDTA precipitation. Sequencing was performed on an ABI 3730 automated sequencer (Applied BioSystems). Sequence editing and alignment using Geneious® 5 (Drummond et al. 2009) was trivial since only one three-basepair (bp) insertion for COII and one three-bp insertion for WG were found.

Analyses

Uncorrected genetic distances (uncorrected p) were calculated using PAUP* (Swofford 2002) and visually depicted with heat maps using a Visual Basic script in Microsoft Excel® to assess the range of genetic distances between species and to distinguish genetic outliers (*i.e.* potential disagreement between taxonomy and genetic distance). Phylogenetic trees were generated using 2579 bp of sequence data from specimens representing 66 Hawaiian *Cydia* and 11 non-Hawaiian *Cydia*, and 12 non-*Cydia* tortricid moths, with *Clepsis peritana* (Clemens) (Tortricinae: Archipini) as the outgroup taxon (Table 1). Phylogenetic estimation criteria included maximum parsimony (MP) and Bayesian (BA) Markov Chain Monte Carlo (MCMC) analyses. Unweighted, unpartitioned MP analysis was performed using TNT (Goloboff et al. 2008) for each gene separately and all genes fragments combined using the new technology search option employing sectorial search, ratcheting, drift, and tree fusing. Two independent runs of 100 replicates with 10 random addition sequences contributed to the final strict consensus tree for each analysis, with 100 random addition bootstrap replicates to measure branch support. Bayesian analysis was performed by MrBayes (Huelsenbeck and Ronquist 2001) on CIPRES (Miller et al. 2009) for all data combined and for each gene fragment separately. Separate analysis of mtDNA and nDNA data followed a GTR model based on recommendations from ModelTest (Posada and Crandall 1998), and allowed bp frequencies for each codon position to vary independently using a partitioned dataset with nst=6 and rates=invgamma for each partition. The 28S analysis was run as a single partition with nst=6 and rates=propinv. For each analysis two independent runs of four chains each were run for 10,000,000 generations, with sampling every 1,000 generations. For each analysis a consensus tree was generated after discarding the first 25% of samples as burnin. Analyses of combined data included all partitions as listed above in a single analysis and combining partitions for mtDNA and nDNA. Combined analyses with partitions required 35,000,000 generations for convergence and adequate mixing of chains as estimated by split frequencies, potential scale reduction factors (PSRF), and tree mixing overlay plots. Branch support was assessed by posterior probability (PP). Bayesian consensus trees were then used to test alternative evolutionary models of progression rule and ecological speciation.

Model Testing

Patterns of diversification within Hawaiian *Cydia* were tested by comparing Bayes factors derived from an unconstrained consensus tree (null model) with alternative hypotheses based on host-plant use and contemporary distribution (Kass and Raftery 1995, Nylander et al. 2004). To compare alternative hypotheses, phylogenies of the Hawaiian species were generated by Bayesian analysis using *Cydia latiferreana* as the outgroup and the following constraint trees: 1) in order to test the progression rule, whereby older lineages are found on older islands, a constraint tree forced monophyly for species found on progressively younger islands (*i.e.* derived from older-island species); and 2) in order to test if shifting to a new host acted as a "key innovation" (Ehrlich and Raven 1964, Berenbaum et al. 1996) that promoted subsequent speciation, constraint trees were constructed that forced species that feed on the same host plant to be either a clade or grade. Bayes factors from the constrained analyses were then compared with the unconstrained analysis. Bayes factors within one or two units indicate that there is no discernable difference between models, while a difference in Bayes factors greater than ten units indicates that the model with the greater Bayes factor (i.e. less negative) is "very strong" evidence in favor of that model (Kass and Raftery 1995, Nylander et al. 2004).

RESULTS

DNA Sequencing

Five gene fragments were successfully sequenced for specimens of Hawaiian *Cydia*, non-Hawaiian *Cydia*, and non-*Cydia* Tortricidae. However, the nDNA genes EF1α and WG failed to amplify for some non-Hawaiian specimens (Table 1). The mtDNA genes, COI and COII, were A/T-rich (70% and 76%, respectively) compared to the rRNA gene, 28S, (48%), while the nDNA genes, WG and EF1α, tended toward greater C/G content (64% and 57%, respectively). Overall, COI and COII had a comparable proportion of variable loci: 34% of 658 bp for COI, and 40% of 480 bp for COII. However, COII showed a far greater proportion of non-synonymous substitutions (24%) than COI (6%). This corresponds with proportionally greater first and second codon position substitutions for COII than for COI (Table 5). Within Hawaiian *Cydia* both COI and COII showed proportionally less variation than the overall trends (18% each), with COII continuing to show a greater proportion of non-synonymous substitutions (Table 5). The 28S gene fragment showed little variation (5 of 520 bp) within Hawaiian *Cydia.* Three variable loci within 28S were limited to one specimen (*walsinghami*_56004), while the two other variable positions had widespread pyrimidine transitions. The 28S fragment did, however, show greater variation (38 of 520 bp) across the entire taxa set. The nDNA gene fragments, particularly WG, also showed considerable variation among outgroup species, and some informative variation within Hawaiian *Cydia* (Table 5, Figure 2).

The within and among species genetic distance for Hawaiian *Cydia* was greatest for COI followed by COII (Table 6). Within species distance (uncorrected P) ranged from 0 – 1.98% for COI, while among species distance ranged from 1.4 – 6.4%. *Cydia mauiensis* was the most different (4.6 – 6.4%) from the remaining Hawaiian *Cydia* species, while the least distance between species (1.4%) was between a specimen of *C. falcifalcella* and *C.*

conspicua. Consistent across all genes was the genetic similarity between *C. rufipennis* and *C. montana*. Within species genetic distance of COI within the *C. rufipennis-montana* complex, treated as two separate species in this analysis, ranged from 0.8 – 0.9% and 0.2 – 1.5% for *C. rufipennis* and *C. montana*, respectively, while the between species distance ranged from 2.28 – 2.74%. Some taxa (*C. mauiensis*, *C. velocilimitata*, *C. falsifalcella*, *C. parapteryx*, *C. hawaiiensis*, *C. koaiae*, *C. latifemoris*) were represented by one to few specimens and are known from only one island (and in some cases were collecting during a single event), while others (*C. conspicua*, *C. makai*, *C. pseudanomalosa*, *C. plicata*, and *C. walsinghamii*) were represented by more specimens and/or were collected from two or more islands.

Phylogenetic Analyses

Cladograms from each of the five genes analyzed separately provided differing levels of resolution and somewhat conflicting topologies. Therefore, each gene tree is presented separately along with a combined-analysis tree. Maximum parsimony and Bayesian topologies were largely in agreement for each gene. Therefore, only Bayesian phylograms are presented because they provide estimates of branch lengths and were used for model testing. Each of the mtDNA and nDNA gene trees strongly support (1.00 PP) the monophyly of a Hawaiian *Cydia* clade, while the 28S tree does not distinguish Hawaiian *Cydia* from other Grapholitini (Figures 3-7). The mtDNA genes, COI and COII, place most of the Hawaiian species in a polytomy with some sister-species relationships evident (Figures 3-4). Both genes recover a sister-relationship between *C. rufipennis* and *C. montana* (> 0.99 PP). Other recovered clades appear particular to the genes analyzed, although the following weak patterns bear mentioning as they gain support in the analysis of the full data-set. COI weakly groups the *Canavalia*-feeding species *C. parapteryx*, *C. falsifalcella*, and *C. velocilimitata* as a clade (0.54 PP) with a weak sister-relationship to the *Acacia*-feeding *C. conspicua* (0.69 PP), but does not include the *Canavalia*-feeding *C. mauiensis*. COI also weakly groups the *C. rufipennis-montana* complex with *C. makai* (0.68 PP). And whereas COI places *C. koaiae* sister to *C. walsinghamii* (0.56 PP), COII places *C. acaciavora* sister to *C. walsinghamii* (0.56 PP). The nDNA gene trees separate the four *Canavalia*-feeding species (*C. mauiensis, C. parapteryx, C. falsifalcella, C. velocilimitata*) from the remaining species, forming a basal polytomy along with *C. conspicua* and *C. koaiae* as sister to the other species in the case of EF1α, and as a separate clade (0.99 PP) within a broader polytomy for WG. Neither gene provides much resolution for the remaining Hawaiian species (Figures 6-7).

The combined analysis for all five genes provides greater resolution across the Hawaiian *Cydia* clade (Figure 8). The *Canavalia*-feeding species form a moderately supported clade (0.76 PP) sister to the remaining Hawaiian species (0.93 PP). The strict consensus maximum parsimony tree (not shown) supports a basal grade of these species, with *C. mauiensis* basally divergent to a clade of the other three species, which in turn are sister to the remaining species. The remaining species form a progression of nested polytomies of varying support (Figures 8-9), with the following notable sister group relationships: *C. haleakalaensis* sister to *C. latifemoris* (0.79 PP); *C. koaiae* sister to *C. conspicua* (0.89 PP); *C. rufipennis* sister to *C. montana* (1.00 PP); and *C. acaciavora* sister to *C. walsinghamii* (0.99 PP).

Model testing and evolutionary patterns

Model testing using Bayes factors does not support a progression rule pattern of phylogeography, but does support successive host-plant shifts (Table 7). Constraining species from younger islands to be nested within older-island species resulted in a topology (not shown) that did not fit the data as well as the unconstrained analysis (107 log likelihood units difference) which scattered older island species throughout the Hawaiian *Cydia* clade (Figure 9). Further evidence against a progression rule pattern is the placement of *C. mauiensis* from Maui as sister-group to the rest of the Hawaiian species in many analyses.

Constraining clades by host-plant affinities, however, resulted in topologies that fit the data as well as unconstrained analyses (Table 7). Constraining species that feed on either *Canavalia*, *Sophora*, or *Acacia* to be monophyletic resulted in a topology (not shown) that fit the data almost as well as the unconstrained analysis (1.99 log likelihood units difference). Constraining each host group to successive nesting (*i.e. Acacia* feeders nested within *Sophora* feeders and together nested within *Canavalia* feeders) resulted in a topology indistinguishable from the unconstrained analysis (0.57 log likelihood units difference).

Origins of Hawaiian *Cydia*

The relationship of Hawaiian *Cydia* to other species sampled is equivocal. The individual-gene and combined-data analyses consistently place the Hawaiian species well within *Cydia* with six non-Hawaiian species, including *C. latiferreana*, clustered as nearest relatives (Figures 3-8). However, none of these species consistently emerged as sister to the Hawaiian species, but rather they grouped more consistently with each other with varying levels of support. *Cydia pseudomalesana* from French Polynesia consistently grouped with *C. undosa* from the Indian Ocean for each gene separately and in combined-analyses (> 95% PP for each). Overall, the *Cydia* species analyzed nested within the tribe Grapholitini with the exception of *C. deshaisiana*, which grouped with species in the tribe Eucosmini.

DISCUSSION

Utility of molecular characters

Five gene fragments provided varying levels of phylogenetic resolution. The two mitochondrial genes, COI and COII, had the greatest amount of variation and consistently united morphologically determined species. However, these genes failed to resolve relationships among most of the Hawaiian species. COII had a greater proportion of non-synonymous first and second codon position changes resulting in more amino acid changes than in COI. COII also resulted in different sister-species pairings than COI for the few species that showed this level of resolution. The "barcode" region of COI (Folmer et al. 1994) used in the present study, therefore, was useful for assigning specimens to species using reciprocal monophyly but not for relationships among species. Furthermore, the 2-3% COI divergence threshold recommended for many animal groups (e.g. Hebert et al. 2004) could result in misidentification of species such as *C. conspicua* and *C. falsifalcella* (1.4 % divergence), consistent with the caution noted by other authors (e.g. Meyer and Paulay 2005) that arbitrary barcode thresholds are not appropriate for understudied taxa.

The nuclear rRNA gene 28S contained little phylogenetically informative variation for the species studied, while the two nuclear genes, EF1α and wingless, provided resolution for among-species relationships. The 28S gene fragment from the 'A' domain used in this study showed variation at the tribe and subfamily level that may be useful for resolving relationships at these higher levels. However, its lack of variation below the tribal level marginalized its utility in the current study. Similarly, EF1α and wingless were of limited value in assigning individuals to species, but variation in these genes among the basal Hawaiian and deeper nodes provided phylogenetic resolution not provided by the mitochondrial genes. Therefore, the combination of characters provided resolution throughout the phylogeny that the individual genes alone could not.

An examination of morphological characters does not refute the molecular phylogeny presented here. *Cydia* males have relatively simplified genitalia, (lacking developed socii, gnathos, or uncus), compared to other Lepidoptera. Hawaiian *Cydia* is also relatively uniform in the proportional size of the wings and legs (Oboyski Chapter 2). One species, *C. anomalosa*, is an exception among the morphological uniformity of the Hawaiian species, calling into question its phylogenetic placement. *Cydia anomalosa* is typical of non-Hawaiian *Cydia* species in having the anal margin of male hindwings rolled dorsally enclosing specialized scales, characteristics suggested as synapomorphic for the genus *Cydia* (Danilevsky and Kuznetsov 1968, Brown and Miller 1983, Komai 1999, Komai and Horak 2006). No other *Cydia* in Hawaii possesses the anal roll, but instead most species possess a ventral pouch on the male hindwing below the cubital vein (along the path of CuP) containing modified cubital pecten scales, much like *C. latiferreana*, *C. maackiana*, and a few other species to a lesser degree (Brown 1983). However, both the mitochondrial and nuclear genes analyzed, together or separately, placed the morphologically divergent *C. anomalosa* well within Hawaiian *Cydia*. It appears that these secondary sexual characters are fairly labile, questioning their value as sources of synapomorphies for this genus (Brown 1983, Brown and Miller 1983).

<u>*Cydia* in the Pacific</u>

Cydia pseudomalesana Clarke (1986) from the Marquesas and Society archipelagos evidently represents a colonization into the Pacific islands separate from the Hawaiian clade. Of the species analyzed, *C. pseudomalesana* pairs consistently with *C. undosa,* reared from *Sophora denudata* Bory from Reunion Island, for all genes analyzed. The larvae of *C. pseudomalesana* in the Marquesas and Society islands feed on the seeds of *Dodonaea viscosa* Jacq. (Sapindaceae) (Oboyski, unpublished data), a cosmopolitan plant that is locally common on islands from Australia to Hawaii (West 1984). Recently, Komai & Horak (2006) reported a *Cydia* species ("sp. A") reared from *D. viscosa* in Australia that appears morphologically similar to *C. pseudomalesana*. Although specimens of the Australian species were not obtained for this study, it is likely these are close relatives, if not the same widespread species. Extensive efforts to rear tortricid larvae from *D. viscosa* seeds in Hawaii (Oboyski and The Nature Conservancy 1997, Oboyski et al. 2001) have resulted in only *Cryptophlebia illepida* (Butler). Therefore, the morphological, molecular, geographic, and behavioral differences between *C. pseudomalesana* and Hawaiian *Cydia* confirm that these two lineages are separate and distinct penetrations into the Pacific.

Several other Pacific Islands tortricid species are currently classified as *Cydia*. Little is known about *C. callizona* (Meyrick) from New Guinea. According to Clarke (1976), *C. celiae* (Clarke) and *C. doria* (Clarke) from Micronesia most closely resemble Indian species of *Cydia*. However, female *C. celiae* lack a diverticulum on the corpus bursae, while male *C. doria* have a somewhat developed uncus (see figures in Clarke 1976), calling into question the generic placement of these Micronesian species. According to Diakonoff (1967), *C. inflata* (Meyrick) from the Philippines is of questionable generic placement. Of the six species known from Japan (*C. infausta* (Walsingham), *C. japonensis* Kawabe, *C. kamijoi* (Oku), *C. kurokoi* (Amsel), *C. pactolana yasudai* (Oku), and *C. trasias* (Meyrick)), only *C. trasias*, reared from the seeds of *Sophora japonica* L., was available for inclusion in this study. Also included in the analyses were *Acanthoclita balanoptycha* (Meyrick) and *A. defensa* (Meyrick) from Micronesia, both considered by Clarke (1976) to be *Cydia* species (Diakonoff 1982). Considering these taxonomic uncertainties, as well as the long branch from the phylogenetic analyses, it is unlikely that any of the species above are close relatives of Hawaiian *Cydia* and do not provide evidence for island hopping to Hawaii. For the present, therefore, the origins of Hawaiian *Cydia* remain obscured.

Hawaiian *Cydia* origins and patterns of diversification

Hawaiian *Cydia* appears to represent a single endemic radiation restricted to the current "high islands" of Hawaii (Hawaii, Maui, Molokai, Oahu, Kauai). The relatively long branch separating the Hawaiian species from other *Cydia* suggests that this genus has a long history in Hawaii. However, the long branch is more likely an artifact of outgroup sampling. The genus *Cydia* currently includes 231 named species and subspecies with a worldwide distribution (Oboyski Chapter 1, Brown *et al.* 2005, Komai and Horak 2006). A few named species are known to both the New World and Old World tropics and Australia, although some of these regions have not been explored to the same extent as the temperate regions. Outgroups used in the present study include *Cydia* and other tortricid species from California, Mississippi, Japan, French Polynesia, Micronesia, Portugal, and Reunion Island, including two *Cydia* that feed on *Sophora* spp. (*C. trasias* and *C. undosa*) and one (*C. latiferreana*) with a pronounced male hindwing pouch superficially similar to Hawaiian *Cydia* (Oboyski Chapter 2, Brown 1983). Phylogenetic analyses placed six species, including *C. latiferreana* and *C. undosa*, near Hawaiian *Cydia*, but these six tended to group closer to each other than to the Hawaiian species, with the arrangement of species differing for each gene analyzed. The lack of agreement among analyses as to which species is most closely related to Hawaiian *Cydia* suggests that none is particularly close, resulting in a long branch to the Hawaiian clade.

Relationships among Hawaiian *Cydia* suggest a relatively recent arrival to Maui Island, with a geological date of 1.2 Mya or less (Price and Clague 2002). Relationships among the Hawaiian species were largely unresolved for individual genes analyzed separately, although a fairly well-supported and resolved phylogeny emerged from the full data set (Figure 8). *Cydia mauiensis*, collected in association with *Canavalia* along the Maui coast, appears basally divergent to taxa from Hawaii Island, Oahu, and Kauai in the sister clade to all other Hawaiian taxa in both maximum parsimony and Bayesian analyses of the full dataset. The early diverging positions of this Maui species and *C. falsifalcella* from Hawaii Island, and those of other taxa from Maui and Hawaii in successively diverging clades of

non-*Canavalia*-feeders, precludes a progression rule of speciation from older to younger islands (Figure 9). Moreover, the progression rule was not supported by analysis of constraints placed on the data to simulate a progression rule pattern.

This study does suggest, however, that speciation accompanied successive colonization of new host-plant genera (Figure 9). Larvae of Hawaiian *Cydia* are confined to three major host-plant genera (*Acacia*, *Canavalia*, and *Sophora*) and two minor host genera (*Strongylodon* and *Vicia*) in the family Fabaceae (Oboyski Chapter 2, Swezey 1954, Zimmerman 1978). None of these genera is endemic to Hawaii and each belongs to a different plant tribe, although each has evolved endemic species in Hawaii (Wagner et al. 1999). Therefore, *Cydia* did not track the diversification of their host-plants after arriving in Hawaii. Rather it appears likely that switching to new host genera acted as a key innovation (sensu Berenbaum et al. 1996, Schluter 2000) that promoted speciation within Hawaiian *Cydia*. Phylogenetic analyses place the *Canavalia*-feeding species most basally divergent in the Hawaiian clade, either as a clade or as a grade with *C. mauiensis* as the earliest diverging lineage. A subsequent shift to feeding on *Sophora chrysophylla* was accompanied by speciation and filling of this feeding niche across the islands. Another shift to *Acacia*-feeding appears to have accompanied another wave of speciation and filling of this niche across the islands (Figure 9).

Despite the distribution of *Canavalia* throughout the Pacific, including widespread coastal species and upland island endemics (Sauer 1964, St. John 1970), no *Cydia* species has been recorded from *Canavalia* outside of Hawaii (Brown et al. 2008). Nor do Hawaiian species appear to share a recent common ancestor with other *Sophora*- or *Acacia*-feeding species. Therefore, *Cydia* immigrants to Hawaii probably had to overcome the defenses of native plant species. Hawaiian species of *Canavalia* have not been assayed for canavanine, a toxic amino acid found in other species of *Canavalia* (Bell 1972), or for other toxic compounds. Apart from native *Cydia*, only a small number of non-native Anthribidae, Bruchidae, and Tortricidae have been reared from *Canavalia* seeds (Oboyski unpublished data), although it is unclear whether this is due to host-plant chemistry or a lack of generalist seed predators. *Sophora chrysophylla* seeds, however, are high in pyralizidine alkaloids (Banko et al. 2002a). Apart from one invasive species of anthribid beetle (Oboyski unpublished data), *S. chrysophylla* seeds are fed on by only endemic Hawaiian *Cydia* and palila birds, *Loxioides bailleui* Oustalet (Banko et al. 2002a). We can assume, therefore, unique physiological adaptations to feeding on these underutilized resources allowed *Cydia* species to spread rapidly across the islands.

Although Hawaiian *Cydia* appears constrained to feeding on species of Fabaceae, the three major host-plant genera are fed on by the same or closely related *Cydia* species on each island (Table 4). For example, *C. koaia*, an *Acacia* twig-feeder on Hawaii Island is sister to the twig-feeding *C. conspicua* found on the older islands. Similarly, the *Acacia* flower-feeding *C. rufipennis* of Kauai and Oahu is sister to the *Acacia* flower-feeding *C. montana* on Maui and Hawaii Islands. However, not all sister pairs are found on different islands. For example, *C. haleakalaensis* is sister to *C. latifemoris*, both *Sophora*-feeding Maui species, and *C. acaciavora* from Maui is sister to the widespread and polymorphic *C. walsinghamii*. A similar island-by-host-plant matrix of herbivorous Hawaiian insects was first noted for

cerambycid beetles (Gressitt 1978), but is obvious for many genera of herbivorous insects with endemic radiations where host-plant affinities are well-known (e.g. Swezey 1954, Asquith 1995, Roderick 1997, Polhemus 2002). As an increasing number of robust phylogenies are generated for Hawaiian insect radiations we can expect the interplay between geography and host-plant in promoting speciation to reveal some general patterns.

Southwood (1960) noted that the diversity of herbivores on Hawaiian trees was directly related to the relative abundance of each tree species, with *Metrosideros* and *Acacia*, the two most common and widespread tree species in Hawaii, supporting the greatest diversity of herbivores (see also Southwood 1961). Likewise, *Acacia*-feeding *Cydia* have the greatest number of species, both within and among islands (Table 2). Two species for which host affinities are unknown, *C. obliqua* and *C. storeella*, likely fed on *Sophora chrysophylla*, given the habitats from which they were collected, making *Sophora*-feeders the second most diverse. *Canavalia*, although not as abundant as the other two host plants, likely were more prominent in Hawaiian forests and waysides in the past, but are particularly vulnerable to browsing by ungulates and are now rare outside of protected areas (St. John 1970, 1972). The herbivore diversity host-plant abundance hypothesis is further supported by the addition of three *Sophora*-feeding *Cydia* on Hawaii Island and Maui, where the abundance of *Sophora*, rare on the other islands, is greatly increased by the addition of the subalpine habitat. And indeed, two *Sophora* seed-feeding *Cydia* species, *C. plicata* and *C. makai*, appear to differ only in their altitudinal limits, with *C. plicata* reaching peak abundance in the subalpine *Sophora* forests of Mauna Kea, Hawaii and Haleakala, Maui and *C. makai* found across all the high islands at low elevations (Oboyski Chapter 2).

Extinction may have played a role in the current distribution of species, although its signal is not obvious in the present analyses. If *Cydia* species existed at high elevations (> 3000 m) on the older islands, these earlier lineages would have gone extinct as those islands eroded and subsided. Such species might have been closer to the original forms that colonized the archipelago than any of the currently known species and might belong to a more basal position in the phylogeny. Several species of Hawaiian *Cydia*, including *C. chlorostola*, *C. crassicornis*, *C. gypsograpta*, *C. obliqua*, and *C. storeella*, each known from one to three individuals collected at the turn of the 20th century, may have gone extinct in recent times (Walsingham 1907, Zimmerman 1978). However, some of these "species" may be members of other more variable species such as *C. plicata* or *C. walsinghamii* (Oboyski Chapter 2). The phylogeny presented here is a hypothesis based on currently available data. Increased sampling, including better outgroup representation, inclusion of extinct species, and more sophisticated analyses can further refine this phylogeny in the future. However, the importance of host-shifting and ecological opportunity in the radiation of Hawaiian *Cydia* is unlikely to be discounted.

CONCLUSIONS

Hawaiian *Cydia* appears to represent a single radiation in the Hawaiian Islands. However, the outgroup taxa used in this analysis provide little insight into the ancestral habits or origins of Hawaiian *Cydia*. A much larger analysis including better representation of Asian and American species is necessary to resolve the likely origins of this group. Given the

positions of *C. mauiensis* and *C. falsifalcella* in all phylogenetic analyses, and the early diverging positions of other Maui and Hawaii Island taxa in the larger clade exclusive of the *Canavalia*-feeding group, *Cydia* appears to have initially colonized Maui or Hawaii Island. Patterns of diversification do not follow a progression rule of speciation from older to younger islands, but do support the hypothesis of successive host-plant shifting from *Canavalia* to *Sophora* to *Acacia* associated with the formation of new species and filling of ecological niches across the high island chain. Geographic isolation does appear to have played an important role in that nominal sister pairs are often found on different islands. This host-plant-by-island matrix pattern is not unusual for herbivorous insects in Hawaii and indicates the importance of both ecology and geography in diversification of vagile, host-seeking, Hawaiian endemics.

Table 1. List of specimens used for molecular phylogeny analysis. General location (Country, US State, or Island) is given for all specimens; particular location (Region) is only given for Hawaiian *Cydia*. All specimens were collected by the author except Mississippi specimens collected along with R.L. Brown, *C. succedana* collected by Q. Paynter, *C. trasias* collected by F. Komai, and *C. undosa* collected by L. Jauze. Specimens were reared from host plants except where noted by superscript[1], which indicates that the host plant is assumed from published records and/or the habitat from which the specimen was collected.

Taxon	Specimen	Location	Host
Tortricinae			
Archipini			
Clepsis peritana (Clemens, 1860)	606.09	Mississippi	at light
Olethreutinae			
Olethreutini			
Episimus argutana (Clemens, 1860)	606.03	Missisiippi	at light
Enarmoniini			
Ancylis burgessiana (Zeller, 1875)	606.15	Mississippi	at light
Eucosmini			
Crocidosema sp.	505.17	Hawaii	at light
Eccoptocera n.sp.	505.05	Hawaii	at light
Epiblema abruptana (Walsingham, 1879)	605.11	Mississippi	at light
Retinia gemistrigulana (Kearfott, 1905)	606.07	Mississippi	at light
Rhopobota finitimana (Heinrich, 1923)	606.01	Mississippi	at light
Grapholitini			
Acanthoclita balanoptycha (Meyrick, 1910)	670.10	Micronesia	at light
Acanthoclita defense (Meyrick, 1922)	688.11	Micronesia	at light
Cryptophlebia ombrodelta (Lower, 1898)	688.14	Micronesia	at light
Cydia caryana (Fitch, 1856)	606.02	Mississippi	at light
Cydia conspicua (Walsingham, 1907)	351.02	Kauai (Kokee)	*Acacia*
Cydia conspicua (Walsingham, 1907)	470.03	Kauai (Kokee)	*Acacia*
Cydia conspicua (Walsingham, 1907)	482.01	Kauai (Kokee)	*Acacia*
Cydia conspicua (Walsingham, 1907)	560.01	Oahu (Waianae Mts)	*Acacia*
Cydia conspicua (Walsingham, 1907)	560.03	Oahu (Waianae Mts)	*Acacia*
Cydia cupressana Kearfott, 1907	621.01	California	*Cupressus*
Cydia deshaisiana (Lucas, 1858)	256.09	California	*Sebastiana*
Cydia falsifalcella (Walsingham, 1907)	596.01	Hawaii (Mauna Loa)	*Canavalia*[1]
Cydia latifemoris Walsingham, 1907)	133.17	Maui (Haleakala)	*Sophora*
Cydia latifemoris (Walsingham, 1907)	133.34	Maui (Haleakala)	*Sophora*
Cydia latifemoris (Walsingham, 1907)	523.02	Maui (Haleakala)	*Sophora*
Cydia latiferreana (Walsingham, 1879)	601.01	California	*Quercus*[1]
Cydia montana (Walsingham, 1907)	226.03	Hawaii (Mauna Kea)	*Acacia*[1]
Cydia montana (Walsingham, 1907)	529.01	Maui (Haleakala)	*Acacia*[1]
Cydia montana (Walsingham, 1907)	573.02	Hawaii (Hualalai)	*Acacia*[1]
Cydia montana (Walsingham, 1907)	584.03	Hawaii (Mauna Loa)	*Acacia*[1]
Cydia parapteryx (Meyrick, 1932)	563.01	Oahu (Waianae Mts)	*Strongylodon*[1]
Cydia parapteryx (Meyrick, 1932)	563.02	Oahu (Waianae Mts)	*Strongylodon*[1]
Cydia parapteryx (Meyrick, 1932)	563.03	Oahu (Waianae Mts)	*Strongylodon*[1]
Cydia plicata (Walsingham, 1907)	097.09	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	108.19	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	112.01	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	124.06	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	133.21	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	135.02	Hawaii (Mauna Kea)	*Sophora*

Table 1. (Continued)

Cydia plicata (Walsingham, 1907)	136.09	Hawaii (Mauna Kea)	*Sophora*
Cydia plicata (Walsingham, 1907)	157.08	Hawaii (Mauna Loa)	*Sophora*
Cydia plicata (Walsingham, 1907)	157.10	Hawaii (Mauna Loa)	*Sophora*
Cydia plicata (Walsingham, 1907)	278.22	Hawaii (Mauna Loa)	*Sophora*
Cydia plicata (Walsingham, 1907)	303.07	Hawaii (Mauna Kea)	*Sophora*
Cydia plicata (Walsingham, 1907)	536.01	Maui (Haleakala)	*Sophora*
Cydia plicata (Walsingham, 1907)	593.01	Hawaii (Hualalai)	*Sophora*
Cydia plicata (Walsingham, 1907)	594.04	Hawaii (Hualalai)	*Sophora*
Cydia pomonella (Linnaeus, 1758)	448.04	California	*Malus*
Cydia prosperana (Kearfott, 1907)	608.01	California	*Pseudotsuga*[1]
Cydia pseudomalesana Clarke, 1986	650.09	Marquesas Islands	*Dodonaea*
Cydia rufipennis (Butler, 1881)	512.01	Kauai (Kokee)	*Acacia*[1]
Cydia rufipennis (Butler, 1881)	515.01	Kauai (Kokee)	*Acacia*[1]
Cydia rufipennis (Butler, 1881)	515.02	Kauai (Kokee)	*Acacia*[1]
Cydia succedana (Denis & Schiffermüller, 1775)	599.02	Portugal	*Ulex*
Cydia trasias (Meyrick, 1928)	600.01	Japan	*Sophora*
Cydia undosa (Diakonoff, 1957)	449.05	Reunion Island	*Sophora*
Cydia walsinghamii (Butler, 1882)	054.01	Hawaii (Hualalai)	*Acacia*
Cydia walsinghamii (Butler, 1882)	201.03	Molokai (Kamiloloa)	*Acacia*
Cydia walsinghamii (Butler, 1882)	226.04	Hawaii (Mauna Kea)	*Acacia*
Cydia walsinghamii (Butler, 1882)	268.01	Oahu (Koolau Mtns.)	*Acacia*
Cydia walsinghamii (Butler, 1882)	470.17	Kauai (Kokee)	*Acacia*
Cydia walsinghamii (Butler, 1882)	509.03	Kauai (Kokee)	*Acacia*
Cydia walsinghamii (Butler, 1882)	529.04	Maui (Haleakala)	*Acacia*
Cydia walsinghamii (Butler, 1882)	560.02	Oahu (Waianae Mts)	*Acacia*
Cydia walsinghamii (Butler, 1882)	560.04	Oahu (Waianae Mts)	*Acacia*
Cydia walsinghamii (Butler, 1882)	570.01	Hawaii (Kilauea)	*Acacia*
Cydia koaiae Oboyski, 2011	323.02	Hawaii (Kohala)	*Acacia*
Cydia hawaiiensis Oboyski, 2011	218.02	Hawaii (Mauna Loa)	*Acacia*[1]
Cydia hawaiiensis Oboyski, 2011	238.08	Hawaii (Kilauea)	*Acacia*[1]
Cydia hawaiiensis Oboyski, 2011	280.04	Hawaii (Mauna Loa)	*Acacia*[1]
Cydia haleakalaensis Oboyski, 2011	521.01	Maui (Haleakala)	*Sophora*[1]
Cydia anomolosa Oboyski, 2011	129.08	Maui (Haleakala)	*Acacia*[1]
Cydia anomolosa Oboyski, 2011	513.04	Kauai (Mt. Kahili)	*Acacia*[1]
Cydia anomolosa Oboyski, 2011	513.08	Kauai (Mt. Kahili)	*Acacia*[1]
Cydia mauiensis Oboyski, 2011	544.07	Maui (East Shore)	*Canavalia*[1]
Cydia mauiensis Oboyski, 2011	544.08	Maui (East Shore)	*Canavalia*[1]
Cydia acaciavora Oboyski, 2011	095.20	Maui (Haleakala)	*Acacia*[1]
Cydia acaciavora Oboyski, 2011	149.01	Hawaii (Kohala)	*Acacia*[1]
Cydia acaciavora Oboyski, 2011	343.05	Maui (Haleakala)	*Acacia*[1]
Cydia makai Oboyski, 2011	153.01	Hawaii (Kohala)	*Sophora*
Cydia makai Oboyski, 2011	154.04	Hawaii (Kilauea)	*Sophora*
Cydia makai Oboyski, 2011	154.05	Hawaii (Kilauea)	*Sophora*
Cydia makai Oboyski, 2011	197.05	Molokai (Kamakou)	*Sophora*
Cydia makai Oboyski, 2011	197.28	Molokai (Kamakou)	*Sophora*
Cydia makai Oboyski, 2011	277.01	Hawaii (Mauna Kea)	*Sophora*
Cydia makai Oboyski, 2011	464.03	Kauai (Kokee)	*Sophora*[1]
Cydia makai Oboyski, 2011	466.06	Kauai (Kokee)	*Sophora*
Cydia velocilimitata Oboyski, 2011	519.01	Kauai (North Shore)	*Canavalia*[1]
Cydia velocilimitata Oboyski, 2011	519.03	Kauai (North Shore)	*Canavalia*[1]
Ecdytolopha mana (Kearfott, 1907)	605.06	Mississippi	at light

Table 2. *Cydia* host-plant relationships by island. The epithet of each Hawaiian *Cydia* species is given for each of three host plant genera on each of the main Hawaiian Islands. Note that moth species may be found on more than one island, but are only listed for one host plant genus [a] (specimens of *C. parapteryx* have also been reared from *Strongylodon ruber*, and *C. falsifalcella* from *Vicia menziessii*). [b] indicates seed-feeding in herbarium specimens, [c] indicates questionable/uncertain distribution status, and [d] indicates the species may require synonymization with another. The five species with unknown host plants are known from one to three individuals each collected 1896-1909.

Island / Host	***Canavalia***	***Sophora***	***Acacia***	**Host Unknown**	**Total # spp.**
Kauai	*C. velocilimitata*	*C. makai*	*C. conspicua* *C. anomalosa* *C. rufipennis* *C. walsinghami*	---	**6**
Oahu	*C. parapteryx* [a]	[b, c]	*C. conspicua* *C. rufipennis* *C. walsinghami*	*C. chlorostola* *C. gypsograpta*	**6**
Molokai	[b, c]	*makai*	*C. walsinghami*	---	**2**
Lanai	[c]	[b, c]	[c]	---	**c**
Kahoolawe	---	---	---	---	**---**
Maui	*C. mauiensis*	*C. latifemoris* *C. plicata* *C. haleakalaensis*	*C. acaciavora* *C. conspicua* *C. montana* *C. anomalosa* *C. walsinghami*	*C. storeella* [d]	**10**
Hawaii	*C. falsifalcella* [a]	*C. latifemoris* [c] *C. makai* *C. plicata*	*C. hawaiiensis* *C. koaiae* *C. montana* *C. walsinghami*	*C. crassicornis* *C. obliqua* [d]	**10**
Total # spp.	**4**	**4**	**8**	**5**	**21**

Table 3. Five gene fragments and associated primers.

Region	# bp	Primer	Primer sequence (5' → 3')	Reference
COI	658	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	*Folmer et al*. (1994)
		HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	
COII	477	Eva	GAG ACC ATT ACT TGC TTT CAG TCA TCT	Caterino & Sperling (1999)
		Strom	TAA TTT GAA CTA TYT TAC CNG CA	
28S	520	28Sa	GAC CCG TCT TGA AGC ACG	
		S8Sr5d2	CCA CAG CGC CAG TTC TGC TTA	
EF1α	518	M13-rcM4	TGT AAA ACG ACG GCC AGT ACA GCV ACK GTY TGY CTC ATR TC	T. Gilligan (tortricid.net)
		M13REV_M51.9tort	CAG GAA ACA GCT ATG ACC CAR GAY GTN TAC AAA ATC GG	
WG	400	LepWG1	GARTGYAARTGYCAYGGYATGTCTGG	Brower & DeSalle (1998)
		LepWG2a	ACTICGCARCACCARTGGAATGTRCA	

Table 4. PCR Reactions (volume and concentration of reagents) for five gene fragments.

Gene Region	**COI, COII, 28S**		**LepWG, EF1α**	
Reagents	µL	[rxn]	µL	[rxn]
dH_2O	8.8	-	5.9	-
10x buffer*	3	-	3	-
$MgCl_2$ (25 mM)	2	2.5 mM	2.5	3.125 mM
BSA (0.1 x)	1	0.1 x	1	0.05
Betaine (1 x)	-	-	3	0.15 x
dNTPs (8 µM)	1	0.4 µM	1	0.4 µM
F primer (10 µM)	1	0.5 µM	0.75	0.375 µM
R primer (10 µM)	1	0.5 µM	0.75	0.375 µM
Taq (5 U)	0.2	0.05 U	0.2	0.05 U
DNA template	2		2	
Total volume	20		20	

*500 mM KCL, 100 mM Tris–HCL at pH 8.3, 15 mM

Table 5. Patterns of genetic variation. Basepair (bp) frequencies for all 89 Tortricidae (All) and 68 Hawaiian *Cydia* (Hawaii) specimens, for five genes (COI, COII, 28S, WG, EF1α). Numbers indicate number of loci, numbers in parentheses indicate percentage. The percentage of parsimony-informative loci (Inform.) is out of the total number of loci. Note that Variable (Var) and Constant (Const.) loci sum to "Total"; synonymous (Syn) and Non-synonymous (Non.) substitutions do not always sum to "Var" because some loci had both synonymous and non-synonymous substitutions; and 1st, 2nd, and 3rd codon positions sum to either "Syn" or "Non" for each column. Ambiguous or polymorphic loci for WG and EF1α were treated as synonymous if one of the possible bases would result in a synonymous substitution.

	COI		COII		28S		WG		EF1α		Total	
	All	Hawaii	All	Hawaii	All	Hawaii	All	Hawaii	All	Hawaii	All	Hawaii
Total	658 bp	658 bp	480 bp	477 bp	520 bp	520 bp	403	400 bp	518 bp	518 bp	2579 bp	2573 bp
Inform.	181 (28)	90 (14)	142 (30)	70 (15)	22 (4)	2 (0.4)	111 (28)	18 (5)	74 (14)	14 (3)	530 (21)	194 (8)
Const.	437 (66)	538 (82)	290 (60)	391 (82)	480 (92)	515 (99)	247 (61)	341 (85)	383 (74)	461 (89)	1837 (71)	2246 (87)
Var.	221 (34)	120 (18)	187 (40)	86 (18)	40 (8)	5 (1)	156 (39)	59 (15)	135 (26)	57 (11)	739 (29)	327 (13)
Syn.	203	116	143	70			114	59	130	56	590	301
1st	28	14	12	6			7	3	3	4	50	27
2nd	0	0	0	0			0	0	5	4	5	4
3rd	175	102	131	64			107	56	122	48	535	270
Non.	13	4	44	16			42	0	5	1	84	21
1st	7	2	29	10			18	0	2	0	56	12
2nd	4	2	12	5			14	0	1	1	31	8
3rd	2	0	3	1			10	0	2	0	17	1
A	(30)	(30)	(36)	(36)	(25)	(25)	(21)	(21)	(23)	(23)	(27)	(27)
C	(16)	(16)	(13)	(13)	(22)	(22)	(31)	(31)	(32)	(32)	(22)	(22)
G	(14)	(14)	(11)	(11)	(30)	(30)	(33)	(33)	(25)	(25)	(22)	(22)
T	(40)	(40)	(40)	(40)	(23)	(23)	(15)	(15)	(20)	(20)	(29)	(29)
A/T	(70)	(70)	(76)	(76)	(48)	(48)	(36)	(36)	(43)	(43)	(56)	(56)
C/G	(30)	(30)	(24)	(24)	(52)	(52)	(64)	(64)	(57)	(57)	(44)	(44)

Table 6. Genetic variation (percent difference) within and among Hawaiian *Cydia* species. Within and among species genetic distance (uncorrected P x 100) for Hawaiian *Cydia* for five gene fragments separately and combined (all).

	Within Species of Hawaiian *Cydia*				**Among Species of Hawaiian *Cydia***			
	Range	Mode	Median	Mean ± SE	Range	Mode	Median	Mean ± SE
COI	0.00 – 1.98	0.91	0.91	0.84 ± 0.03	1.37 – 6.38	2.89	3.19	3.34 ± 0.02
COII	0.00 – 1.89	0.63	0.63	0.59 ± 0.03	0.42 – 6.29	3.35	3.35	3.51 ± 0.02
28S	0.00 – 0.58	0.00	0.00	0.03 ± 0.01	0.00 – 0.77	0.19	0.19	0.14 ± 0.00
WG	0.00 – 0.51	0.00	0.00	0.12 ± 0.01	0.00 – 2.50	0.50	0.51	0.79 ± 0.01
EF1α	0.00 – 0.97	0.00	0.00	0.16 ± 0.02	0.00 – 1.37	0.58	0.58	0.47 ± 0.01
All	0.00 – 1.47	0.61	0.55	0.54 ± 0.01	0.73 – 4.45	2.09	2.41	2.46 ± 0.01

Table 7. Models tested using Bayes factors. Comparison of alternative models using Bayes factors. The log likelihood functions ($\log_e f(X|M)$) are given for a null model (M_0 – an unconstrained consensus tree) and an alternative model (M_1 – phylogeny constrained by host or distribution). "Three host clades" forced monophyly for species feeding on each of three host plant genera. "Three host grades" constrained the topology to a progression of host plant feeding from *Canavalia* to *Sophora* to *Acacia*. "Younger islands nested" constrained Maui and Hawaii Island-limited species to a clade (***i.e.*** Nested within the older islands). * $2\log_e B_{10} < 2$ indicates the two models being compared are indistinguishable; $2\log_e B_{10} > 10$ is a "very strong" indication that the model with the higher likelihood function (i.e. less negative) is a better fit (see Kass and Raftery 1995, Nylander et al. 2004).

Model comparison (M_1/M_0)	$\log_e f(X\|M_1)$	$\log_e f(X\|M_0)$	$\log_e B_{10}$	$2\log_e B_{10}$
Three host clades / unconstrained	**-6818.18**	**-**6816.19	1.99	3.98
Three host grades / unconstrained	**-6816.76**	-6816.19	0.57	1.14*
Younger islands nested / unconstrained	-6922.97	**-6816.19**	107	214

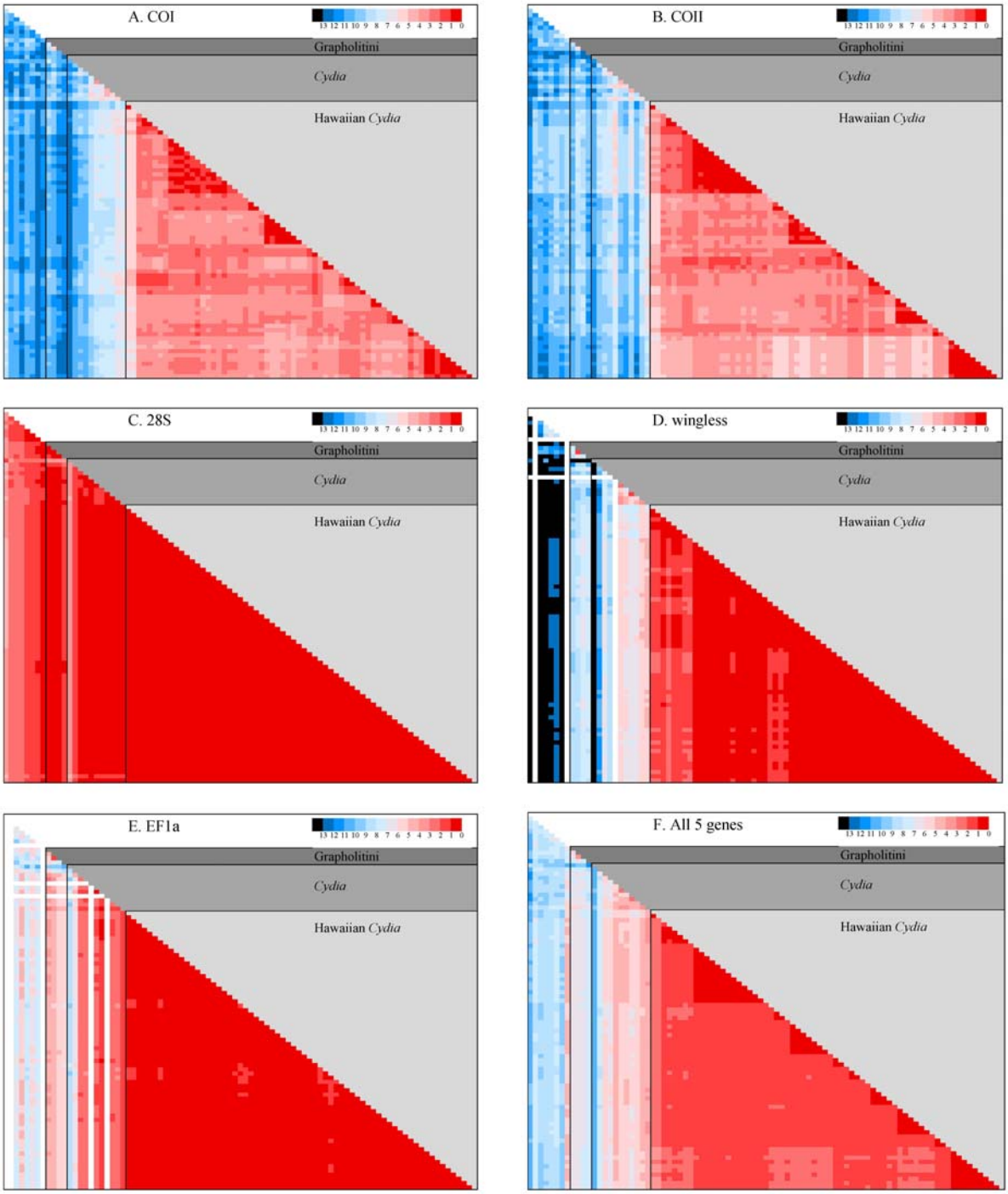

Figure 2. Heat maps – genetic distances between tortricid species. Visual depiction of genetic distance to illustrate the range of genetic variation at different taxonomic scales for five genes separately (A-E) and all genes combined (F). Color scale = uncorrected p distance x 100, with deep red indicating virtually identical genotypes to black indicating maximum genetic distance (>13% difference, missing data in white). Note that the combined map (F) "smooths" the erratic variation of the individual genes and distinguishes genetic outliers for each taxonomic group (*i.e.* conspicuously different heat color than neighboring species). Order of taxa along the left and bottom axes follows the order of specimens in Figure 8, except *Cydia deshaisiana* is in the first *Cydia* position. The diagonal (*i.e.* each specimen compared to itself) is not displayed.

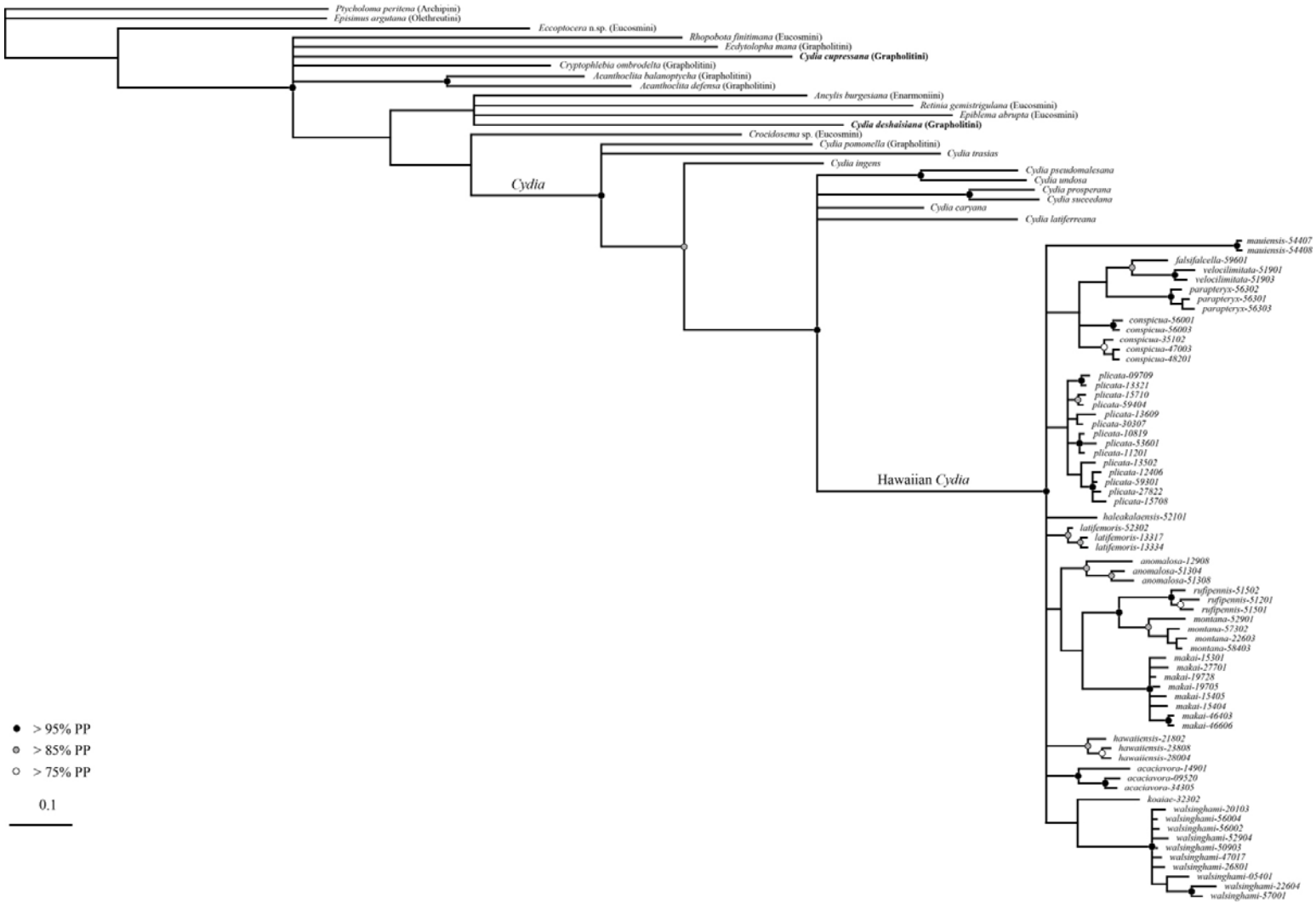

Figure 3. Gene tree for COI. Bayesian partitioned analysis (GTR model, nst=6, rates=invgamma) allowing each codon position to vary independently. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

Figure 4. Gene tree for COII. Bayesian partitioned analysis (GTR model, nst=6, rates=invgamma) allowing each codon position to vary independently. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

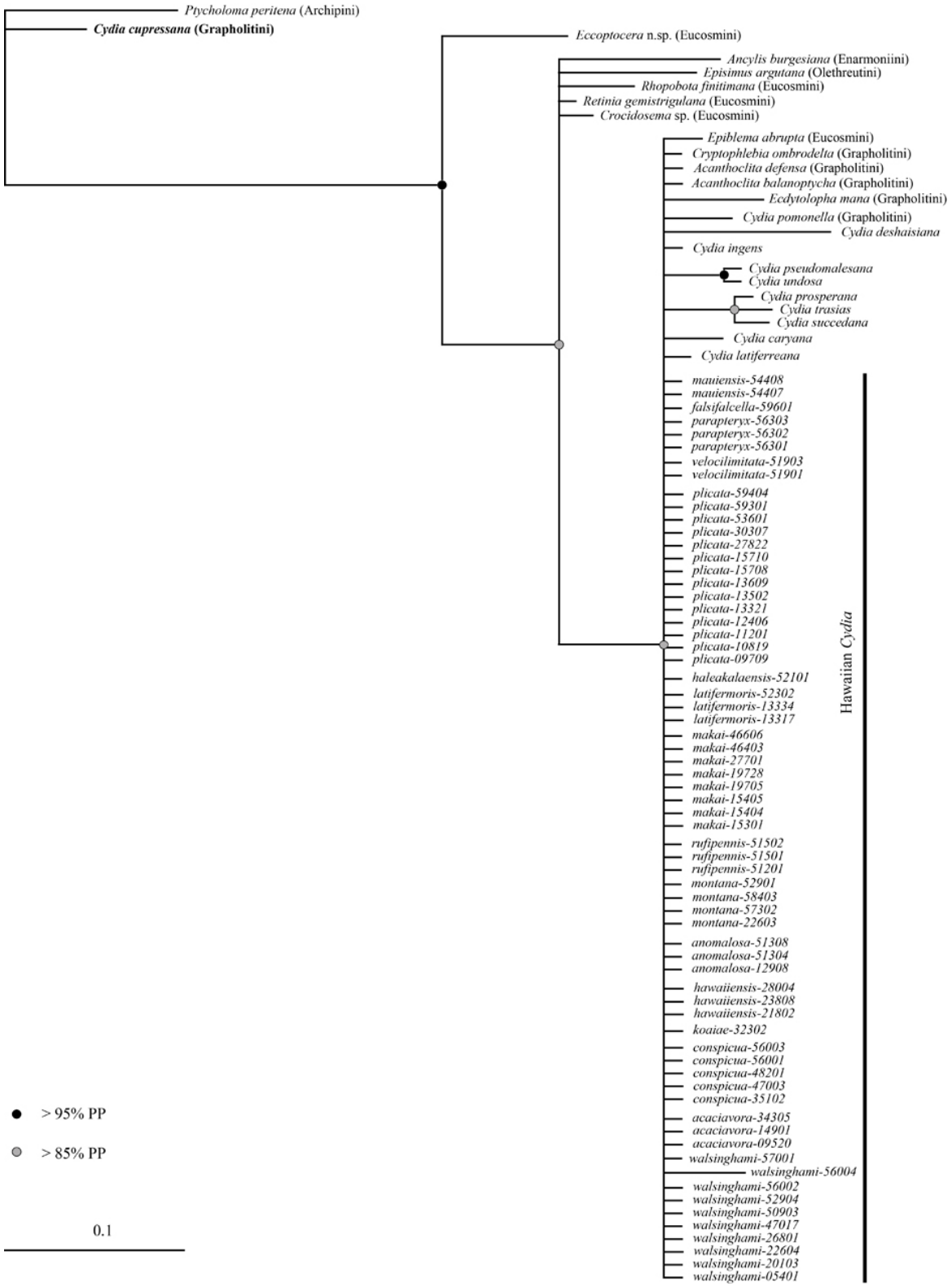

Figure 5. Gene tree for 28S. Bayesian partitioned analysis (nst=6, rates=propinv) with no partitions. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

Figure 6. Gene tree for wingless (WG). Bayesian partitioned analysis (GTR model, nst=6, rates=invgamma) allowing each codon position to vary independently. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

Figure 7. Gene tree for Elongation Factor 1 α (EF1α). Bayesian partitioned analysis (GTR model, nst=6, rates=invgamma) allowing each codon position to vary independently. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

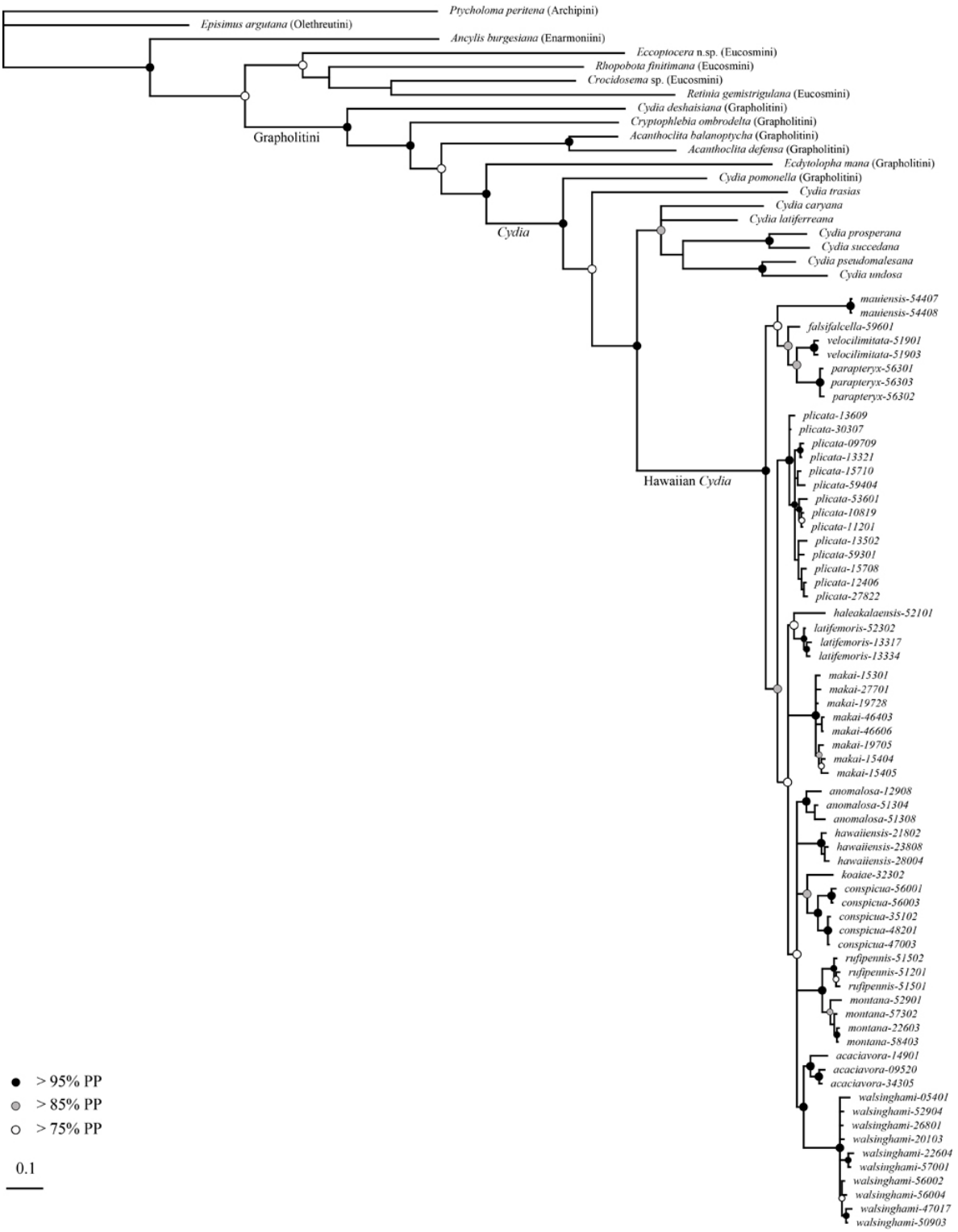

Figure 8. Phylogeny using five genes combined (COI, COII, 28S, WG, EF1α). Bayesian partitioned analysis (GTR model, nst=6, rates=invgamma) allowing each codon position to vary independently for mtDNA and nDNA; and (nst=6, rates=propinv) with no partitions for 28S. Branch length scale bar indicates expected number of basepair changes. Black, gray, and white circles indicate node support. Nodes without circles have less than 75% posterior probability support.

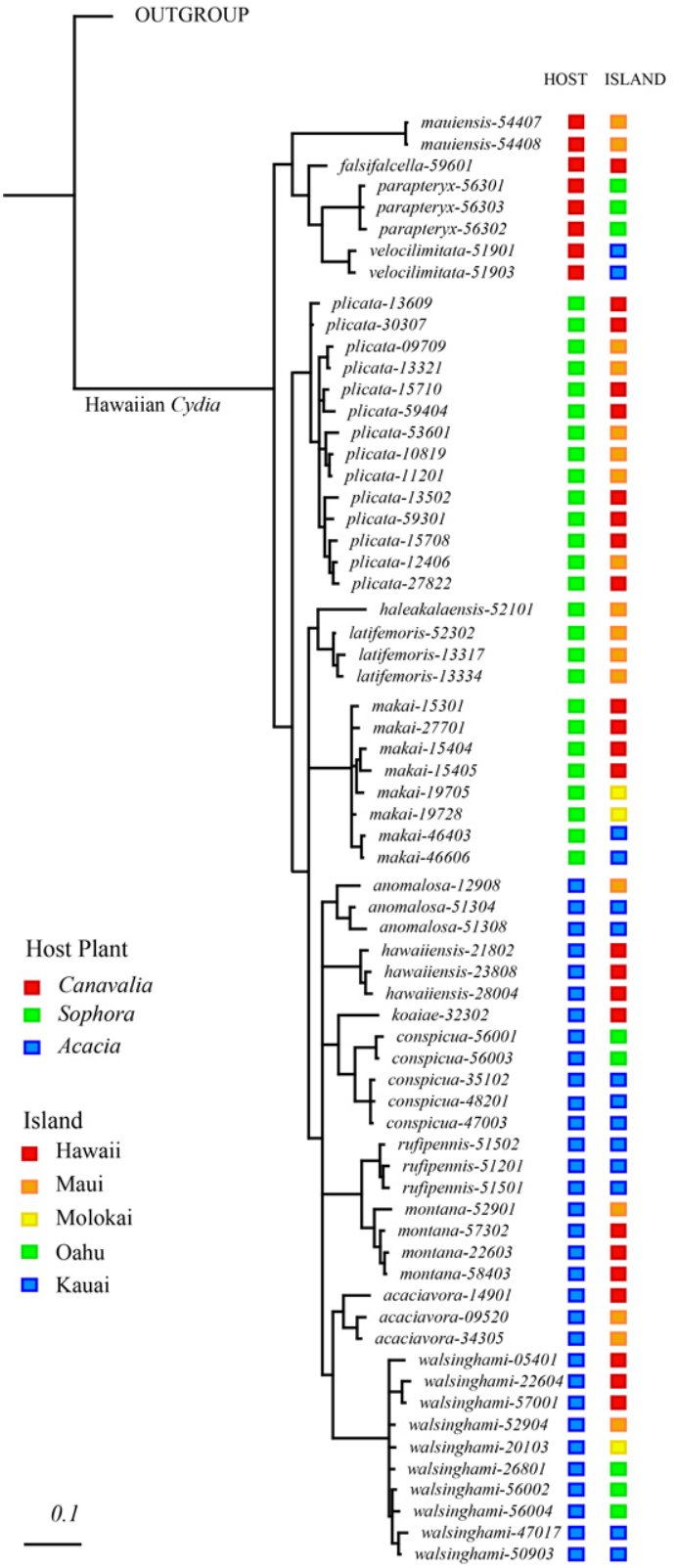

Figure 9. Phylogeny, host-plant affinities, and distribution for Hawaiian *Cydia*. Bayesian reconstruction using five gene fragments (see previous figure) with outgroups collapsed to a single branch, and each specimen color coded for host-plant and island origin. Scale bar indicates expected number of basepair changes.

REFERENCES CITED

Asquith, A. 1995. Evolution of *Sarona* (Heteroptera, Miridae): Speciation on geographic and ecological islands. Pages 91-120 *in* D. L. Wagner and D. J. Funk, editors. Hawaiian biogeography: Evolution on a hot spot archipelago. Smithsonian Institution Press, Washington.

Banko, P. C., M. L. Cipollini, G. W. Breton, E. Paulk, M. Wink, and I. Ishaki. 2002a. Seed chemistry of *Sophora chrysophylla* (mamane) in relation to the diet of the specialist seed predator *Loxioides bailleui* (palila) in Hawaii. Journal of Chemical Ecology 28(7):1393-1410.

Banko, P. C., P. T. Oboyski, J. W. Slotterback, S. J. Dougill, D. M. Goltz, L. Johnson, M. E. Laut, and T. C. Murray. 2002b. Availability of food resources, distribution of invasive species, and conservation of a Hawaiian bird along a gradient of elevation. Journal of Biogeography 29:789-808.

Bell, E. A. 1972. Toxic Amino Acids in the Leguminosae. Pages 163-177 *in* J. B. Harborne, editor. Phytochemical Ecology. Academic Press, New York.

Berenbaum, M. R., C. Favret, and M. A. Schuler. 1996. On defining "key innovations" in an adaptive radiation: Cytochrome P450S and Papilionidae. The American Naturalist 148:S139-S155.

Berlocher, S. H. and J. L. Feder. 2002. Sympatric speciation in phytophagous insects: moving beyond contraversy. Annual Review of Entomology 47:773-815.

Brenner, G. J., P. T. Oboyski, and P. C. Banko. 2002. Parasitism of *Cydia* spp. (Lepidoptera: Tortricidae) on *Sophora chrysophylla* (Fabaceae) along an elevation gradient of dry subalpine forest on Mauna Kea, Hawaii. Pan-Pacific Entomologist 78:101-109.

Brower, A. V. Z. and R. DeSalle. 1998. Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of wingless as a source of characters for phylogenetic inference. Insect Molecular Biology 7:73-82.

Brown, J. W., J. Baixeras, R. L. Brown, M. Horak, F. Komai, E. H. Metzler, J. Razowski, and K. R. Tuck. 2005. Tortricidae (Lepidoptera). Pages 1-741 *in* J. W. Brown, editor. World Catalogue of Insects, Volume 5. Apollo Books, Stenstrup.

Brown, J. W., G. S. Robinson, and J. A. Powell. 2008. Food plant database of the leafrollers of the world (Lepidoptera: Tortricidae) (Version 1.0.0).

Brown, R. L. 1983. Taxonomic and morphological investigations of Olethreutinae: *Rhopobota*, *Griselda*, *Melissopus*, and *Cydia* (Lepidoptera: Tortricidae). Entomography 2:97-120.

Brown, R. L. and P. R. Miller. 1983. Studies of Lepidoptera hindwings with emphasis on ultrastructure of scales in *Cydia caryana* (Fitch) (Tortricidae). Entomography 2:261-295.

Caterino, M. S. and F. A. H. Sperling. 1999. Papilio phylogeny based on mitochondrial cytochrome oxidase I and II genes. Molecular Phylogenetics and Evolution 11(1):122-137.

Clarke, J. F. G. 1976. Microlepidoptera: Tortricoidea. Insects of Micronesia 9(1):1-144.

Clarke, J. F. G. 1986. Pyralidae and microlepidoptera of the Marquesas Archipelago. Smithsonian Contributions to Zoology 416:1-485.

Danilevsky, A. S. and V. I. Kuznetsov. 1968. Tortricidae Laspeyresiini. Fauna SSSR. Lepidopterous Insects Series 7, 5(1):1-635.

Despres, L. and M. Cherif. 2004. The role of competition in adaptive radiation: a field study on sequentially ovipositing host-specific seed predators. Journal of Animal Ecology 73:109-116.
Diakonoff, A. 1967. Microlepidoptera of the Philippine Islands. United States National Museum Bulletin (Smithsonian Institution) 257:1-484.
Diakonoff, A. 1982. On a collection of some families of microlepidoptera from Sri Lanka (Ceylon). Zoologische Verhandelingen 193:1-124.
Dres, M. and J. Mallet. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. Philosophical Transactions of the Royal Society of London B 357:471-492.
Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, T. Thierer, and A. Wilson. 2009. Geneious v5 <http://www.geneious.com>
Egan, S. P., P. Nosil, and D. J. Funk. 2008. Selection and genomic differentiation during ecological speciation: Isolating the contributions of host-association via a comparative genome scan of *Neochlamisus bebbianae* beetles. Evolution 62:1162–1181.
Ehrlich, P. R. and P. H. Raven. 1964. Butterflies and plants: a study in coevolution. Evolution 18:586-608.
Eldredge, L. G. and H. H. Evenhuis. 2003. Hawaii's biodiversity: A detailed assessment of the numbers of species in the Hawaiian Islands. Bishop Museum Occasional Papers 76:1-29.
Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294-299.
Friar, E. A., J. M. Cruse-Sanders, and M. E. McGlaughlin. 2007. Gene flow in *Dubautia arborea* and *D. ciliolata*: the roles of ecology and isolation by distance in maintaining species boundaries despite ongoing hybridization. Molecular Ecology 16:4028–4038.
Funasaki, G. Y., P. Y. Lai, L. M. Nakahara, J. W. Beardsley, and A. K. Ota. 1988. A review of biological control introductions in Hawaii: 1890 to 1985. Proceedings of the Hawaiian Entomological Society 28:105-160.
Funk, V. A. and W. L. Wagner. 1995. Biogeographic patterns in the Hawaiian Islands. Pages 379-419 *in* W. L. Wagner and V. A. Funk, editors. Hawaiian BIogeography. Evolution on a hot spot archipelago. Smithsonian Institute, Washington.
Gillespie, R. G. and B. G. Baldwin. 2010. Island biogeography of remote archipelagoes. Pages 358-387 *in* J. B. Losos and R. E. Ricklefs, editors. The theory of island biogeography revisited. Princeton University Press, Princeton, NJ.
Gillespie, R. G. and G. K. Roderick. 2002. Arthropods on islands: Colonization, speciation, and conservation. Annual Review of Entomology 47:595-632.
Goloboff, P., J. Farris, and C. Nixon. 2008. TNT, a free program for phylogenetic analysis. Cladistics 24:774-786.
Gressitt, J. L. 1978. Evolution of the endemic Hawaiian cerambycid beetles. Pacific Insects 18(3/4):137-167.
Hebert, P. D. N., M. Y. Stoeckle, T. S. Zemlak, and C. M. Francis. 2004. Identification of Birds through DNA Barcodes. Plos Biology 2:e312.
Howarth, F. G. 1990. Hawaiian terrestrial arthropods: an overview. Bishop Museum Occasional Papers 30:4-26.
Huelsenbeck, J. P. and F. Ronquist. 2001. MRBAYES. Bayesian inference of phylogeny. Bioinformatics 17:754 - 755.

Kass, R. E. and A. E. Raftery. 1995. Bayes factors. Journal of the American Statistical Association 90:773-795.
Koçak, A. O. and M. Kemal. 2007. A replacement name for a Hawaiian moth (Tortricidae, Lepidoptera). Centre for Entomological Studies Miscellaneous Papers 105:3-4.
Komai, F. 1999. A taxonomic review of the genus Grapholita and allied genera (Lepidoptera: Tortricidae) in the Palaearctic region. Entomologica Scandinavica (Supplement) 55:4-226.
Komai, F. and M. Horak. 2006. Grapholitini Guenée. Pages 396-467 *in* M. Horak, editor. Olethreutine moths of Australia. CSIRO Publishing, Canberra.
Matsubayashi, K. W. and H. Katakura. 2009. Contribution of multiple isolating barriers to reproductive isolation between a pair of phytophagous ladybird beetles. Evolution 63(10):2563-2580.
Matsubayashi, K. W., I. Ohshima, and P. Nosil. 2010. Ecological speciation in phytophagous insects. Entomologia Experimentalis et Applicata 134:1-27.
Meyer, C. P. and G. Paulay. 2005. DNA barcoding: Error rates based on comprehensive sampling. Plos Biology 3(12)e422:2229-2238.
Michael, C. S. and S. M. Carolyn. 2010. Multitrait, host-associated divergence among sets of butterfly populations: Implications for reproductive isolation and ecological speciation. Evolution 64:921-933.
Miller, M. A., M. T. Holder, R. Vos, P. E. Midford, T. Liebowitz, L. Chan, P. Hoover, and T. Warnow. 2009. CIPRES. Cyberinfrastructure for phylogenetic research <http://www.phylo.org/sub_sections/portal> Accessed: 2010-12-01. The CIPRES Portals.
Nishida, G. M. 2002. Hawaiian Terrestrial Arthropod Checklist. forth edition. BP Bishop Museum, Honolulu. available online <http://www2.bishopmuseum.org/HBS/checklist/query.asp?grp=Arthropod>.
Nosil, P., S. P. Egan, and D. J. Funk. 2008. Heterogeneous genomic differentiation between walking-stick ecotypes: "Isolation by adaptation," and multiple roles for divergent selection. Evolution 62:316-336.
Nylander, J. A. A., F. Ronquist, J. P. Huelsenbeck, and J. L. Nieves-Aldrey. 2004. Bayesian phylogenetic analysis of combined data. Systematic Biology 53(1):47-67.
O'Grady, P. M., R. T. Lapoint, J. Bonacum, J. Lasola, E. Owen, Y. Wu, and R. DeSalle. 2011. Phylogenetic and ecological relationships of the Hawaiian *Drosophila* inferred by mitochondrial DNA analysis. Molecular Phylogenetics and Evolution 58:244-256.
Oboyski, P. T. and T. N. Conservancy. 1997. Arthropod Survey at Pohakuloa Training Area, Island of Hawaii, Hawaii. Report to US Army Garrison, Hawaii.
Oboyski, P. T., A. J. Gregor, L. B. Passerello, J. P. Weber, J. E. Hines, and P. C. Banko. 2001. Kīpuka Alalā terrestrial arthropod survey, Pōhakuloa Training Area, Hawaii. Final report to US Army Garrison, Hawaii. USGS-BRD, Pacific Island Ecosystems Research Center, Hawaii.
Oboyski, P. T., J. W. Slotterback, and P. C. Banko. 2004. Differential parasitism of seed-feeding *Cydia* (Lepidoptera: Tortricidae) by native and alien wasp species relative to elevation in subalpine *Sophora* (Fabaceae) forests on Mauna Kea, Hawaii. Journal of Insect Conservation 8:229-240.
Peccoud, J. and J.-C. Simon. 2010. The pea aphid complex as a model of ecological speciation. Ecological Entomology 35:119-130.

Perkins, R. C. L. 1913. Introduction. Pages xv-ccxxviii *in* D. Sharp, editor. Fauna Hawaiiensis. Cambridge University Press, Cambridge.
Polhemus, D. A. 2002. An initial review of Orthotylus in the Hawaiian Islands, with descriptions of twenty-one new species (Heteroptera: Miridae). Journal of the New York Entomological Society 110(3-4):270-340.
Posada, D. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817 - 818.
Price, J. P. and D. A. Clague. 2002. How old is the Hawaiian biota? Geology and phylogeny suggest recent divergence. Proceedings of the Royal Society of London Series B-Biological Sciences 269:2429-2435.
Rice, A. M., A. Rudh, H. Ellegren, and A. Qvarnström. 2011. A guide to the genomics of ecological speciation in natural animal populations. Ecology Letters 14:9-18.
Roderick, G. K. 1997. Herbivorous insects and the Hawaiian silversword alliance: Coevolution or cospeciation? Pacific Science 51:440-449.
Roderick, G. K. and R. G. Gillespie. 1998. Speciation and phylogeography of Hawaiian terrestrial arthropods. Molecular Ecology 7:519-531.
Rubinoff, D. 2008. Phylogeography and ecology of an endemic radiation of Hawaiian aquatic case-bearing moths (Hyposmocoma: Cosmopterigidae). Philosophical Transactions of the Royal Society B-Biological Sciences 363:3459-3465.
Sauer, J. 1964. Revision of *Canavalia*. Brittonia 16(2):106-181.
Schluter, D. 2000. The ecology of adaptive radiations. Oxford University Press, Oxford.
Southwood, T. R. E. 1960. The abundance of the Hawaiian trees and the number of their associated insect species. Proceedings of the Hawaiian Entomological Society 17:299-303.
Southwood, T. R. E. 1961. The Number of Species of Insect Associated with Various Trees. Journal of Animal Ecology 30:1-8.
St. John, H. 1970. Revision of the Hawaiian species of *Canavalia* (Leguminosae). Hawaiian Plant Studies. 32. Israel Journal of Botany 19:161-219.
St. John, H. 1972. *Canavalia kauensis* (Leguminosae), a new species from the Island of Hawaii. Pacific Science 26(4):1-6.
Swezey, O. H. 1954. Forest Entomology in Hawaii. An annotated check-list of the insect faunas of the various components of the Hawaiian forests [B.P. Bishop Museum Special Publication 44]. Honolulu Star-Bulletin, Honolulu
Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
Thibert-Plante, X. and A. P. Hendry. 2011. The consequences of phenotypic plasticity for ecological speciation. Journal of Evolutionary Biology 24:326-342.
Thomas, Y., M. T. Bethenod, L. Pelozuelo, B. Frerot, and D. Bourguet. 2003. Genetic isolation between two sympatric host-plant races of the European corn borer, *Ostrinia nubilalis* Hübner. I. Sex pheromone, moth emergence timing, and parasitism. Evolution 57(2):261-273.
Turner, G. F. and M. T. Burrows. 1995. A model of sympatric speciation by sexual selection. Proceedings of the Royal Society of London Series B-Biological Sciences 260:287-292.
Wagner, W. L., D. R. Herbst, and S. H. Sohmer. 1999. Manual of the flowering plants of Hawaii. University of Hawaii Press, Honolulu.

Walsingham, L. 1907. Microlepidoptera. Pages 469-759 *in* D. Sharp, editor. Fauna Hawaiiensis. Cambridge University Press, Cambridge.

West, J. G. 1984. A revision of *Dodonaea* Miller (Sapindaceae) in Australia. Brunonia 7:1-194.

Yoder, J. B., E. Clancey, S. Des Roches, J. M. Eastman, L. Gentry, W. Godsoe, T. J. Hagey, D. Jochimsen, B. P. Oswald, J. Robertson, B. A. J. Sarver, J. J. Schenk, S. F. Spear, and L. J. Harmon. 2010. Ecological opportunity and the origin of adaptive radiations. Journal of Evolutionary Biology 23:1581-1596.

Zimmerman, E. C. 1948. Insects of Hawaii, Volume 1: Introduction. University of Hawaii Press, Honolulu.

Zimmerman, E. C. 1978. Insects of Hawaii, Volume 9: Microlepidoptera, Part I. University of Hawaii Press, Honolulu.