Available online at www.sciencedirect.com
SCIENCE DIRECT®

Molecular Phylogenetics and Evolution 37 (2005) 73–82

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Phylogeny and age of diversification of the *planitibia* species group of the Hawaiian *Drosophila*

James Bonacum [a,*], Patrick M. O'Grady [b], Michael Kambysellis [c], Rob DeSalle [d]

[a] *Department of Biology, University of Illinois at Springfield, HSB 231, Springfield, IL 62703-5407, USA*
[b] *Department of Biology, University of Vermont, 120A Marsh Life Sciences, Burlington, VT 05405, USA*
[c] *Department of Biology, New York University, 1009 Main Building, 100 Washington Square East, New York, NY 10003, USA*
[d] *Department of Invertebrate Zoology, American Museum of Natural History, 79th Street @ Central Park West, New York, NY 10024, USA*

Received 13 October 2004; revised 7 March 2005
Available online 26 April 2005

Abstract

The Hawaiian *Drosophila* offer a unique opportunity to examine evolutionary questions because of the known ages of the Hawaiian Islands and the large number of species endemic to this archipelago. One of the more well studied groups of Hawaiian *Drosophila* is the *planitibia* species group, a long-standing population genetic model system. Here we present a molecular phylogenetic hypothesis of all 17 taxa in the *planitibia* group based on nucleotide sequences from two mitochondrial (16S and COII) and four nuclear (*Adh*, *Gpdh*, *Yp1*, and *Yp2*) loci, accounting for over 4 kb of sequence per taxon. We use these data to estimate major divergence times within this group. Our results suggest that the basal diversification within this group, calculated at 6.1 ± 0.47 MY, predates the oldest high island of Kauai. The older diversifications in this group took place on Kauai, with subsequent colonization and speciation events occurring as new islands became available to *Drosophila*. Understanding of the phylogenetic relationships of this important group will place the existing population genetic work in a macroevolutionary context and stimulate additional work, particularly on those taxa endemic to the Maui Nui complex of islands.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Divergence dates; Hawaiian *Drosophila*; *Planitibia* species group; Biogeography

1. Introduction

The Hawaiian *Drosophila* have served as a model system for the study of adaptive radiation for over 40 years (Craddock, 2000). This is a morphologically diverse clade of an estimated 1000 species, only about 600 of which have been described (Hardy and Kaneshiro, 1981; O'Grady, 2002). At an estimated 26 million years old (Russo et al., 1995) they are also the oldest radiation of plants or animals in the Hawaiian Islands (Price and Clague, 2002). Many species of Hawaiian *Drosophila* are characterized by extreme sexual dimorphism in males, including bizarre modifications of their legs, mouthparts, heads, and/or wings. All species are endemic to the Hawaiian Islands, a remote archipelago roughly 3900 km from the nearest continental land mass. The Hawaiian Archipelago (Fig. 1) is an excellent "natural laboratory" for evolutionary studies because of the diversity and complexity of its ecosystems (Benning et al., 2002; Gillespie, 2004; Hotchkiss and Juvik, 1999; Hotchkiss et al., 2000; Price and Clague, 2002) and endemic flora and fauna (Wagner et al., 1999). The Hawaiian Islands are formed as a volcanic "hot spot" in the ocean floor slowly seeps lava to build large island masses. The ages of all islands have been determined using potassium–argon (K–Ar) dating techniques (Carson and Clague, 1995; Clague and Dalrymple, 1987; Price and Clague, 2002). These islands are arrayed in a more or less linear fashion with the older islands being found in

* Corresponding author. Fax: +1 217 769 206 6162.
E-mail address: jbona1@uis.edu (J. Bonacum).

1055-7903/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2005.03.008

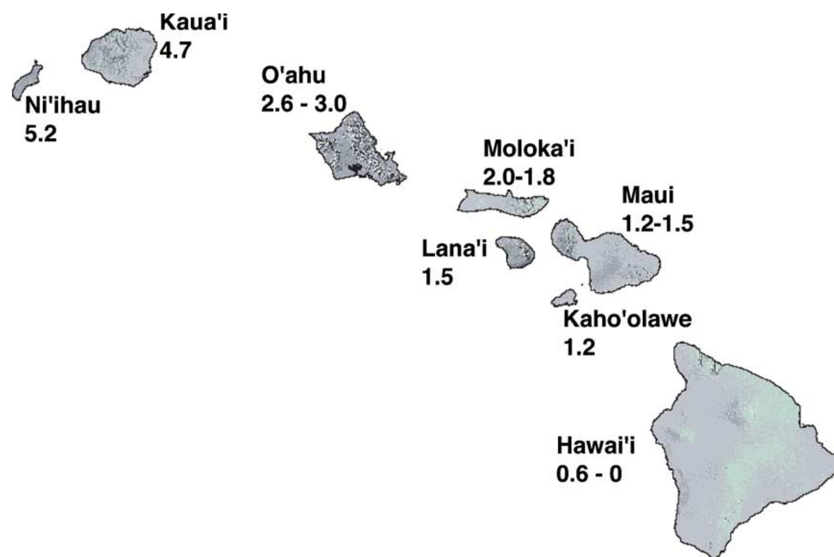

Fig. 1. Map of the Hawaiian islands with the ages of the individual islands as estimated by K–AR dating.

the northwest end of the chain and the younger land masses, like the Big Island of Hawai‘i, found in the southeast. These dates may be used as calibration points for estimating colonization and divergence times between taxa (O'Grady and Zilversmit, 2004; Russo et al., 1995).

The picture wing group, a clade of 112 described species, have been particularly useful as an evolutionary model system (Carson, 2004; Carson and Kaneshiro, 1976; Carson and Templeton, 1984; Kaneshiro, 1997; O'Grady, 2002). The picture wing taxa are placed in five species groups (*primaeva*, *adiastola*, *grimshawi*, *glabriapex*, and *planitibia*). Based upon wing patterns, the morphology of the male genitalia, and polytene chromosome inversions (Carson, 1992; Kaneshiro et al., 1995). Our study concentrates on the *planitibia* species group, and is the first molecular phylogenetic study to include all seventeen species in the group. Although this group is distributed throughout the high Hawaiian Islands all species placed in it are, with one exception, single island endemics. Taxonomically, the *planitibia* group has been quite difficult to circumscribe and, at one time, 15 members of this group were assigned to a separate genus, *Idiomyia*, based on the presence of a supernumerary cross vein. Carson et al. (1967) used polytene chromosome inversions to demonstrate that this character has had multiple origins and that in several cases, species which possess the additional cross vein are more closely related to other species of Hawaiian *Drosophila* which lack this feature than they are to one another. Consequently, *Idiomyia* was synonymized with *Drosophila*. The chromosomal inversion data was also used to generate a phylogeny that placed the *planitibia* species group within a larger picture wing clade (Carson and Stalker, 1968). Additional species were added to that phylogeny in later years (Carson, 1971; Carson and Kaneshiro, 1976; Clayton et al., 1972).

Spieth (1982), in a non-cladistic analysis of mating behaviors, divided the 17 members of the *planitibia* group into five sections (I–V). Within one of these sections he identified two lineages which he designated α and β. The combined analysis of male genitalia and chromosomal inversions by Kaneshiro et al. (1995) identified three species subgroups within the *planitibia* group, *cyrtoloma*, *planitibia*, and *picticornis*. The *cyrtoloma* species subgroup includes all of the α lineage of Spieth as well as five additional species: *D. hanaulae*, *D. obscuripes*, *D. nigribasis*, *D. substenoptera*, and *D. neopicta* which Spieth had assigned to other sections. The *planitibia* species subgroup, as defined by Kaneshiro, contains the same species as Spieth's β lineage. Kaneshiro's *picticornis* species subgroup consists of two species, *D. picticornis* and *D. setosifrons*, which Spieth assigned to his sections I and II, respectively. Although additional support for the monophyly of the *planitibia* species group can be found in various publications (Baker and DeSalle, 1997; Ho, 1994; Ho et al., 1996; Kambysellis et al., 1995; Thomas and Hunt, 1991) none of these studies included all *planitibia* group species.

In the present study, we combine data from two mitochondrial loci (16S rDNA and COII) and four nuclear genes (*Adh* and *Gpdh* from this study and YP1 and YP2 (Ho, 1994; Ho et al., 1996; Kambysellis et al., 1995) with chromosomal inversions (Carson, 1992) to address the phylogenetic relationships within the *planitibia* species group using maximum parsimony. The molecular loci were also analyzed using maximum likelihood and Bayesian methods and, in general, concur with the parsimony analyses of all data. Molecular clock estimates were made using Yoder and Yang's (2000) local clock method to place the evolution of the *planitibia* group in a temporal context. Species distributions were mapped onto the phylogeny to investigate biogeographic

patterns within this group, including the number, direction, and destinations of the migratory events which took place as these species radiated throughout the Hawaiian Islands.

2. Materials and methods

2.1. DNA isolation, amplification, and sequencing

Specimens were collected using standard methods (Carson and Heed, 1986). Flies were either preserved in 100% ETOH or frozen at −80 °C until DNA extractions could be performed. All 17 members of the *planitibia* subgroup are represented in the present study. Outgroup taxa, selected based on previous phylogenetic studies (O'Grady, 2002), include *D. primaeva*, a basal picture-winged species (Kaneshiro et al., 1995), and *D. adunca*, a member of the *antopocerus* species group (Kaneshiro, 1976a,b). Pinned, cryopreserved, and DNA voucher material for all taxa are present in the collections of the American Museum of Natural History and the University of Vermont. Collection information is shown in Appendix 1.

DNA was isolated from individual flies by the method of DeSalle et al. (1993). Four separate gene regions, two mitochondrial (mt), and two nuclear (nu), were amplified with PCR. The mt loci consisted of a 492 base pair (bp) fragment of the 16S rDNA locus and the entire 688 bp cytochrome oxidase II (COII) gene. The nuclear genes we generated sequence for were a 510 bp fragment of the alcohol dehydrogenase (Adh) gene (O'Grady et al., 1998; Russo et al., 1995) and an approximately 1000 bp portion of the α-glycerolphosphate dehydrogenase (Gpdh) gene (Barrio and Ayala, 1997). PCRs were performed in a Perkin Elmer 9600 thermal cycler. The primer sequences used to amplify the 16S, COII, and GPDH gene regions were taken from the following sources: 16S (Wilkinson et al., 1998); COII (Brower, 1994); and GPDH (Barrio and Ayala, 1997). The primers used to amplify ADH were ADHF (5′TY GATTCGCATTGACAAYCCC-3′) and ADHR (5′TTCRATAGSWTCCAGRCGGCC-3′). Although individual primer pairs varied slightly in the annealing temperature and the number of cycles, the general reaction profile consisted of an initial 2 min denaturing step at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 46 °C, and 1 min at 72 °C. An extension step of 7 min at 72 °C was added after the final amplification cycle. PCR products were purified with GeneClean (Bio 101) as per the manufacturer's instructions. Sequencing reactions were performed using the BigDye kit (Applied Biosystems), again following the manufacturer's instructions. The same primers were used for both amplification and sequencing. Sequencing reactions were run on an ABI 377 automated DNA sequencer. Fragments were sequenced in both directions and individual fragments were assembled using Sequencher 3.0 (Gene Codes Corporation 1995). Sequences were verified by visual inspection of the chromatograms. The sequences have been deposited in GenBank (Appendix 1) under the following accession numbers: 16S (AY006389–AY006407), COII (AY006427–AY006445), *Adh* (AY006408–AY006426), and *Gpdh* (AY006446–AY006464). *Yp1* and *Yp2* sequences were taken from GenBank.

2.2. Sequence alignment and phylogenetic analysis

The COII and 16S alignments were trivial as no indels appeared in these sequences. The *Adh* and *Gpdh* sequences were aligned using ClustalX (Thompson et al., 1997) and subsequently checked by eye. We explored a range of gap to change costs (1:1 1:2 1:5 1:10) to identify regions of ambiguous alignment (Gatesy et al., 1993). There was very little effect of weighting scheme on the alignment as the nu gene fragments examined contained very few indels. We also included sequence data for two additional nu genes, Yolk Protein I (Yp1, 994 bp) and Yolk Protein II (Yp2, 372 bp) from previous studies (Ho, 1994; Ho et al., 1996; Kambysellis et al., 1995). Alignments for Yp1 and Yp2 were identical to those published.

Data were analyzed in (a) individual gene partitions, (b) combined mt (16S and COII) and nu (Adh, Gpdh, Yp1, and Yp2) partitions, (c) a combined molecular partition, and (d) a matrix with all molecular plus chromosome inversion characters using maximum parsimony in PAUP* 4.0b10 (Swofford, 2004). The partition homogeneity test (ILD of Farris et al., 1995), as implemented in PAUP* (Swofford, 2004) was performed to assess combinability of partitions from disparate data sources. Although some partitions were significantly incongruent, suggesting that they should not be combined, we combined all data into a simultaneous analysis to fully explore our data. All characters were equally weighted and the search settings were as follows: search type = heuristic; addition sequence = random; number of additions = 500; and branch swapping = TBR. Support for relationships in each maximum parsimony analysis was assessed using bootstrap proportions (BP; Felsenstein, 1978), jackknife (JK; Farris et al., 1996), decay indices (DI; Bremer, 1988, 1992), and partitioned branch support (PBS; Baker and DeSalle, 1997; Baker et al., 1998). Bootstrap settings were as follows: search type = heuristic; addition sequence = random; number of additions = 10; and number of bootstrap replicates = 200. Two hundred jackknife replicates were also performed with percent characters resampled set to 37% following Farris et al. (1996). The program TreeRot (Sorenson, 1999) was used to calculate DI and PBS.

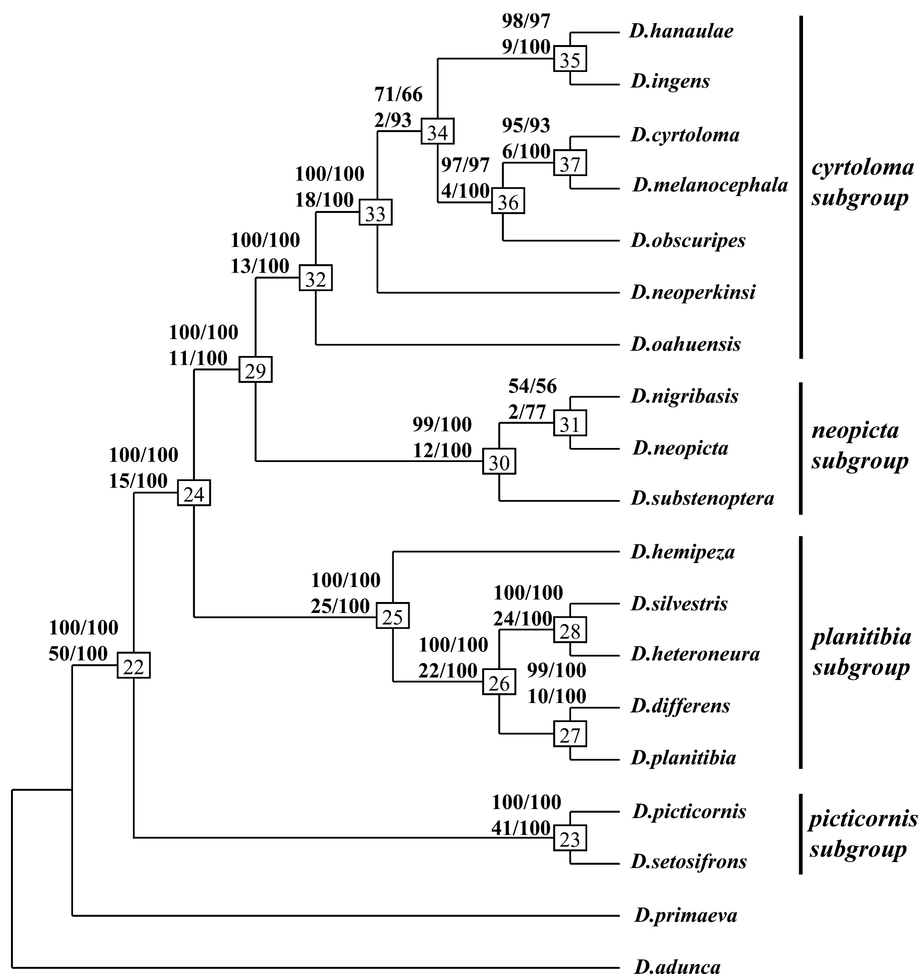

Fig. 2. Phylogenetic tree derived from maximum parsimony analysis of the combined analysis of the molecular (mt and nu) and chromosomal inversion data sets. Numbers above branches indicate bootstrap (left) and jackknife (right) proportions, numbers below branches indicate Bremer support (left) and Bayesian posterior probability values. Numbers refer to specific nodes referenced in the text.

Molecular characters (analyses a–c, above) were also analyzed using maximum likelihood (ML) and Bayesian inference. Modeltest (Version 3.06; Posada and Crandall, 1998) was used to determine optimal models and model parameters for both individual and combined molecular partitions. These models were then used in ML searches with the following settings for individual loci: search type = heuristic; addition sequences = random; number of replicates = 10; and branch swapping = TBR. Combined analyses (all data, nuclear loci, etc.) were done with the above settings but using 100 replicates. Support was assessed using 100 bootstrap replicates (settings as above). Mr. Bayes (Huelsenbeck and Ronquist, 2001) was used to perform Bayesian inference. Models used were the same as those in the ML analyses. Four chains were run simultaneously for 1,000,000 generations. Each chain was sampled every 100 generations and a burnin of either 500 or 1000 was selected, depending on when the chains reached stationarity. A flat prior was used, as per the default settings in Mr. Bayes. Each search was restarted from a random point in search space a total of 10 times to reduce the probability of becoming trapped on local optima. Phylogenetic results and support for each node is shown in Fig. 2.

2.3. Divergence time estimation

Only the combined molecular data (analysis c, above) was used for divergence time estimations. We used a likelihood ratio test to determine whether the combined molecular data fit the hypothesis of a global clock when tested against the assumption of no clock. The null model was rejected at the $P = 0.01$ level (Modeltest 3.06; Posada and Crandall, 1998), suggesting that a global clock is not appropriate for these data. As a result, we used a version of the local clock (Yoder and Yang, 2000) to estimate divergence times within the *planitibia* group. Pairwise relative rate tests (outgroup = *D. adunca* or *D. primaeva*, model = GTR) were performed using HYPHY, version 0.95beta (Kosakovsky-Pond and Muse, 2000) to determine rate classes for various branches (Kress and Specht,

2004). Pairwise comparisons that failed relative rate tests were used in conjunction with ML tree topology to assign various rate classes to nodes in an effort to correct for rate heterogeneity and fit to a local molecular clock (Kress and Specht, 2004; Yoder and Yang, 2000). All branches shown in bold (Fig. 3) were assigned one rate class and all other nodes were given another. Divergence times were estimated in PAML, version 3.13 (Yang, 1997) using multiple calibration points (Price and Elliot-Fisk, 2004), shown in ovals: *D. silvestris*/*D. heteroneura* divergence (node 28, 0.6 MY), *obscuripes* clade (node 36, 1.2 MY), Maui *cyrtoloma* taxa (node 34, 1.5 MY), *D. differns*/*D. planitibia*/*D. silvestris*/*D. heteroneura* divergence (node 26, 2.0 MY), Maui Nui *cyrtoloma* taxa (node 33, 2.0 MY), *planitibia* subgroup (node 25, 2.6–3.0 MY), *neopicta* subgroup (2.6–3.0 MY, as above), and the *cyrtoloma* subgroup (2.6–3.0 MY, as above). In cases where a range of date estimates were available (e.g., O'ahu divergences), both were estimated and an average was used following Jordan et al. (2003). The use of multiple calibration points allows for a more accurate divergence time estimate when using evolutionary rates that are heterogeneous within the taxa being studied. Divergence time estimates at each node are mean values based on eight independent calibration points (above) run in PAML. Time estimates at calibration nodes include only seven values; set calibration times were excluded from mean value calculations (i.e., the *D. silvestris*/*D. heteroneura* calibration value (0.6 MY) was not

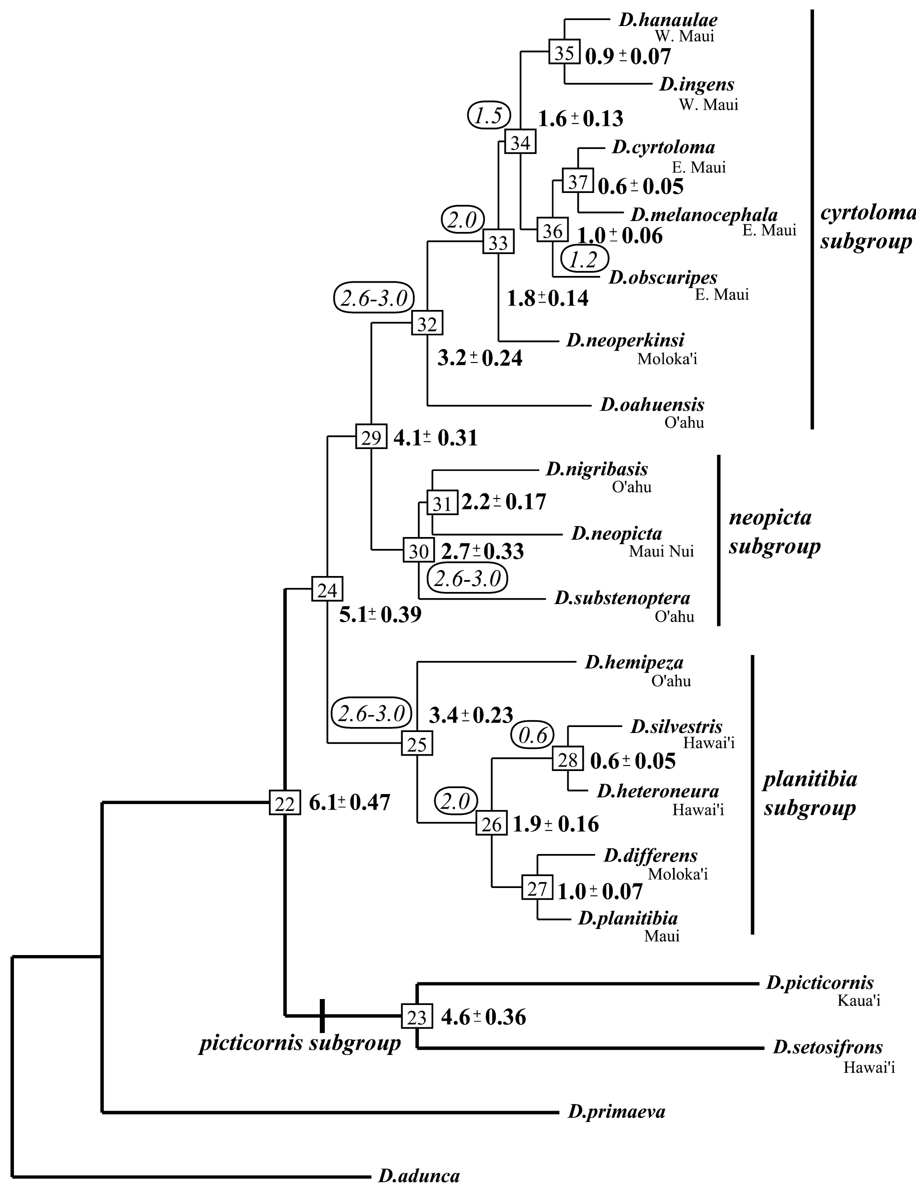

Fig. 3. Results of maximum likelihood analyses of combined data matrix under a GTR + G + I model. Local clock analyses were performed using this phylogeny. Branches in bold were allowed to evolve at one rate, those not in bold were given a separate rate. Multiple calibration points, circled and in italics, are indicated. Bold numbers next to each node are estimated divergence times. Numbers placed in squares are used to mark each node (see text).

used to calculate divergence between *D. silvestris* and *D. heteroneura*, it was based on estimates based on the other seven nodes).

3. Results and discussion

3.1. Phylogenetic analysis

The individual gene partitions and the combined mt and nu data sets yielded trees that were generally in agreement with one another regardless of the optimality criterion employed (data not shown). Individual analyses tend to be less resolved than the large combined mt, nu, and simultaneous analyses, largely because the individual data partitions contain fewer characters. Although a full discussion of all these results is not feasible here, Tables 1 and 2 show summary tree statistics for maximum parsimony and maximum likelihood analyses.

Fig. 2 shows the results of maximum parsimony analysis on a combined molecular and chromosomal data matrix. Maximum likelihood and Bayesian topologies are identical to the combined analysis parsimony tree, even though the chromosomal characters must be excluded in these analyses. Support for each node, based on bootstrap proportions, jackknife, and decay indices (maximum parsimony) and 95% credibility values (Bayesian) are shown (see Section 2). As expected, the Bayesian measures of support are slightly higher than either the parsimony bootstrap proportions or jackknife values (Huelsenbeck and Ronquist, 2001). The maximum parsimony analysis resulted in a single most parsimonious tree of 1429 steps (CI = 0.70, RI = 0.65). The phylogenetic relationships proposed in the current study (Figs. 2 and 3) are largely in agreement with the previous notions of evolutionary relationships in this group (DeSalle and Templeton, 1988; Kambysellis et al., 1995).

Table 1
Summary of maximum parsimony analyses

Partition	No. of trees	Tree length	CI	RI
All data	1	1429	0.700	0.649
mtDNA	5	613	0.519	0.530
nuDNA	6	787	0.849	0.790
Inversions	1	11	1.00	1.00
16S	15	88	0.784	0.743
COII	3	514	0.484	0.521
Adh	7	139	0.842	0.768
Gpdh	32	227	0.855	0.783
Yp1	7	324	0.858	0.814
Yp2	285	86	0.895	0.877

Ensemble consistency (CI) and retention (RI) indices are shown with number of most parsimonious trees and length of shortest tree(s) found.

Table 2
Summary of maximum likelihood analyses

Partition	Model[a]	−ln *L*	G^2	I^3	Base frequencies	Rate matrix
All data, no clock	GTR + G + I	13601.86511	0.7903	0.5249	A = 0.2962	A-C = 1.7101
					C = 0.2168	A-G = 3.5294
					G = 0.2043	A-T = 1.8310
					T = 0.2827	C-G = 1.4947
						C-T = 7.9272
						G-T = 1.0000
All data, enforce clock	As above	136236.58654	As above	As above	As above	As above
16S	HKY + G	1154.70894	0.0152	0	A = 0.3966	A-C = 1.0000
					C = 0.1377	A-G = 3.0114
					G = 0.0922	A-T = 1.0000
					T = 0.3735	C-G = 1.0000
						C-T = 6.8588
						G-T = 1.0000
COII	GTR + G + I	3097.08285	0.5449	0.5817	A = 0.3306	A-C = 1.8981
					C = 0.1610	A-G = 16.4023
					G = 0.1178	A-T = 1.9222
					T = 0.3907	C-G = 1.0038
						C-T = 38.0527
						G-T = 1.0000
Adh	K80 + G	1556.82589	0.5046	0	Equal	TRatio = 1.0101
Gpdh	HKY + G	2833.52607	0.3562	0	A = 0.2829	TRatio = 1.1693
					C = 0.2256	
					G = 0.2111	
					T = 0.2804	
Yp1	K80 + G	3290.62443	0.4100	0	Equal	TRatio = 1.1291
Yp2	K80 + G	1047.25091	0.6704	0	Equal	TRatio = 1.0497

[a] Models of evolution used include: the the Kimura 2-parameter model (K80), where base frequencies are equal but transitions and transversions (TRatio) can have different rates, the HKY model which relaxes the equal base frequency constraint of the K80 model, and the general time reversible model (GTR) that allows for six rate parameters and unequal base frequencies. Additional parameters inferred were the gamma shape parameter (G^2) or the percent of invariant sites (I^3) present.

The *planitibia* species group is strongly supported as monophyletic in our analyses (Fig. 2, node 22). The basal-most split in this clade is represented by the *picticornis* subgroup. Within the *planitibia* group, a number of subgroups are strongly supported (Fig. 2) including the *picticornis* (node 23), *planitibia* (node 25), and *cyrtoloma* subgroups (node 32). The *neopicta* lineage, (node 30) while suggested in a previous study (Kambysellis et al., 1995), is strongly supported in the current study and we propose establishing it as a new subgroup within the larger *planitibia* species group.

In general, the phylogenetic relationships within the individual species subgroups are also well supported. All but two of the nodes on the tree show strong support as measured by BP, JK, and DI values. One of these nodes is found within the *neopicta* species subgroup and supports the sister taxa relationship of *D. nigribasis* and *D. neopicta* (Fig. 2, node 31). The other node with comparatively weak support is found within the *cyrtoloma* species subgroup and supports the monophyly of *D. hanaulae*, *D. ingens*, *D. cyrtoloma*, *D. melanocephala*, and *D. obscuripes* (Fig. 2, node 34). Divergences at both of these nodes have taken place in the Maui Nui complex of islands and may have been the result of recent and rapid diversification events following changes in sea level following the latest glaciation period (Price and Elliot-Fisk, 2004).

3.2. Early temporal and biogeographic patterns

Calculated ages of divergence at each node are shown in bold (Fig. 3). The *planitibia* group (node 22) was formed approximately 6.1 million years ago when the *picticornis* subgroup split off from the remaining taxa. Based on estimates of the past elevations and areas of the northwest Hawaiian Islands (Price and Clague, 2002) we can infer which island(s) the ancestor of the *planitibia* group may have evolved on. Only two islands, Gardner and Necker, had sufficient elevation to support rainforest species within this time frame. Although Gardner, which has been dated at 16 MY was far larger than Necker, with a maximum area greater than 10,000 km^2 and an elevation over 4000 m, by around 6 million years ago it had already begun to subside and most likely contained limited rain forest habitat. Furthermore, it was roughly 800 km from Kaua'i. Necker, dated at 11 MY is a far younger island, and six million years ago was only about 400 km from the still-forming island of Kaua'i (Price and Clague, 2002).

Strict interpretation of the basal position of the *picnsticornis* subgroup, represented by *D. picticornis* on Kaua'i and *D. setosifrons* from Hawai'i, suggests two possible hypotheses. The first is that the ancestor of these taxa was endemic to the Big Island and subsequently "backcolonized" the remainder of the island chain sometime in the course of the past 600,000 years (the maximum estimated age of the Big Island). The second possibility is that the ancestors of these two species diverged on Kaua'i in the distant past and the *D. setosifrons* ancestor colonized the rest of the island chain as new habitats became available. The local clock divergences suggest that the second hypothesis is more likely (Fig. 3). *Drosophila picticornis* and *D. setosifrons* last shared a common ancestor roughly 4.6 million years ago, a time that is in line with the age of Kaua'i, and well before the formation of O'ahu, the next oldest island. This suggests that the ancestors of these two taxa diverged on Kaua'i, and then one lineage dispersed down the island chain yielding the present day distribution.

There are three explanations to account for the absence of any *picticornis* subgroup taxa on the intervening islands: extinction, reduced rates of speciation within this lineage, or incomplete taxon sampling. Incomplete taxon sampling is possible, but highly unlikely due to the extensive sampling efforts by over 150 researchers in the past 45 years (reviewed in O'Grady and Zilversmit, 2004; Spieth, 1982). It is more difficult to differentiate between extinction and reduced rates of speciation. Ecological differences between the *picticornis* subgroup and the remaining *planitibia* species might favor either scenario. For example, the *picticornis* subgroup is linked to sap fluxes on a variety of tree species (Heed, 1968), a condition that would limit resource availability and, as a result, population sizes. These substrates are highly ephemeral and could conceivably reduce speciation rates or increase extinction events. The other *planitibia* species are either specialized on Araliaceae (*cyrtoloma* and *neopicta* subgroups) or Campanulaceae (*planitibia* subgroup), two relatively large clades of endemic Hawaiian plants (Wagner et al., 1999). Both Araliaceae and Campanulaceae have been linked to speciose groups of Hawaiian *Drosophila* (Kambysellis et al., 1995). We favor the extinction hypothesis slightly over lack of speciation because (a) the remainder of the *planitibia* species group seems to form new species readily (Figs. 2 and 3) and many species in the more derived lineages are becoming rare and have nearly been extirpated in the wild (Foote and Carson, 1995). Still, it is clear that additional study is needed to make a more definitive statement.

The basal radiations within the "derived" *planitibia* taxa were estimated at about 5.1 (Fig. 3, node 24) and 4.1 (Fig. 3, node 29) million years ago. This suggests that the main lineages in this group had diversified on Kaua'i (or an even older island) prior to the formation of O'ahu around 3.0 million years ago. The *planitibia* (Fig. 3, node 25), *cyrtoloma* (Fig. 3, node 32) ,and *neopicta* (Fig. 3, node 30) subgroups all diversified between 3.4 and 2.7 million years ago, roughly at the time the island of O'ahu was forming (Fig. 1). This is reasonable given that the basal member of each of these three subgroups is endemic to O'ahu. Subsequent rounds of diversification then took place in each subgroup on islands in the Maui

Nui complex. Within the *cyrtoloma* subgroup, for example, *D. neoperkinsi* diverged on Moloka'i about 1.8 million years ago, followed by a division between East and West Maui species around 1.6 million years ago. Species then evolved in situ on either East or West Maui within the past 1.0 million years. The *planitibia* subgroup shows a similar pattern with an added colonization and diversification on the Big Island by *D. heteroneura* and *D. silvestris* within the past 0.6 million years.

3.3. Diversification on Maui Nui

The most difficult problem in Hawaiian biogeography is reconstructing the history of taxa living on the islands of the Maui Nui complex. The four islands in the complex consist of six separate volcanoes, which, during their formation coalesced into a single island, with the age of the oldest shield volcano estimated at 2.0 million years. Erosional processes, combined with subsidence of the individual volcanoes, and a rise in sea level during the past 300,000–400,000 years have resulted in the fragmentation of Maui Nui into the present day islands of Moloka'i, Maui, Lana'i, and Kaho'olawe. Species found on these islands may have arisen via founder events as they migrated between different volcanoes, or they may have diverged from one another as the result of a vicariance event that occurred when the habitat that once supported a widespread ancestral population became fragmented. Our results indicate that each of the three species subgroups colonized the Maui Nui complex independently (nodes 27, 31, and 33) and subsequently diverged either by vicariance or founder events.

Our data suggests that the *planitibia* subgroup colonized Maui Nui roughly 1.9 million years ago, at about the same time shield formation on Moloka'i was being completed. This ancestral taxon split into two lineages, one of which is endemic to the Big Island and another that is found on Moloka'i and Maui. The Maui species, *D. planitibia*, diverged from its Moloka'i sister taxon, *D. differens*, about 1.0 million years ago, at about the time that Maui was forming. The Big Island species *D. heteroneura* and *D. silvestris* diverged from one another roughly 600,000 years ago, right around the time the Kohala volcano was completing shield formation in the northern part of the Big Island.

A second colonization of Maui Nui, this time by the *neopicta* species subgroup, is represented by a single species, *D. neopicta*. This species is found on both Moloka'i and Maui and is the only member of the *planitibia* species group to inhabit more than a single island. This is a relatively old lineage that dates to roughly 2.2 million years. Lack of subsequent diversification in this subgroup is puzzling and may be due to considerable gene flow between the individual populations on Maui Nui. Additional genetic study of *D. neopicta* populations will be needed to address this question more thoroughly.

The biogeographic history of the *cyrtoloma* species subgroup can be interpreted as a combination of founder and vicariance events. *Drosophila neoperkinsi* first colonized Moloka'i, the oldest island in the Maui Nui complex roughly 1.8 million years ago. The East and West Maui taxa diverged shortly thereafter, about 1.6 million years ago. Whether the ancestral lineage found on Maui resulted from dispersal from Moloka'i or vicariance of a once widespread population found throughout Maui Nui remains unclear. The West Maui ancestor then diversified into two taxa, *D. hanaulae* and *D. ingens*, about 900,000 years ago. The East Maui ancestor formed three species, *D. obscuripes*, *D. cyrtoloma*, and *D. melanocephala*, from 1.0 to 0.6 million years ago. This coincides with intermittent volcanic activity on Haleakala (Price and Elliot-Fisk, 2004). Again, it is difficult to differentiate between dispersal from different volcanic peaks and isolation on different islands as a result of the rise in sea level. Data from mating asymmetry experiments has demonstrated that speciation in the members of the *grimshawi* species group found on Maui Nui did not occur as a result of a series of founder events (Giddings and Templeton, 1983), but these results should not be misinterpreted to mean that founder effects cannot play a role in speciation on Maui Nui. To date, mating asymmetry experiments have not been performed between the various species within the *cyrtoloma* subgroup undoubtedly because these species are difficult to culture.

4. Conclusions

Price and Clague (2002) have recently reviewed the Hawaiian molecular clock literature in light of improved age estimates for the Hawaiian Islands. They find that most of the plant and animal taxa examined date to the age of Kauai (~5 MY) or younger. They suggest that island formation, as well as the availability of higher elevation rainforest habitat might be quite variable over long temporal scales. For example, there was an extended period of time from about 10 to 5 million years ago when there were few high islands with well developed rainforest habitat (Price and Clague, 2002). Only three groups thus far, *Drosophila* (26 MY), Campanulaceae (15 MY), and *Megalagrion* (9.6 MY) predate the formation of Kauai (Price and Clague, 2002).

Examining the temporal and biogeographic history of the major lineages of Hawaiian *Drosophila* will no doubt tell us much about how this group has diversified over time. O'Grady and Zilversmit (2004) examined the *haleakalae* species group and suggest that this clade originated between 9.8 and 10 million years ago, with the majority of diversification taking place after the formation of Kauai. This suggests that, while some of the major lineages of Hawaiian *Drosophila* may have been

extant as long as 10 million years ago, the bulk of species diversity has arisen in the past 5 million years. Studies on other groups of Hawaiian *Drosophila*, as well as other Hawaiian insect taxa, will will lead to a better understanding of the rates, patterns, and processes of species formation in the Hawaiian Islands.

The *planitibia* group, estimated at roughly 6 million years old, predates the oldest current high Island of Kaua'i. This result is in agreement with studies on the *haleakalae* group, suggesting that the major lineages of the Hawaiian *Drosophila* were in place prior to the formation of Kaua'i. The basal radiations within this group, at 5.1 and 4.1 million years ago, as well as the diversification within the basal *picticornis* subgroup, estimated at 4.6 million years ago, all date to Kaua'i. Major diversifications within this group took place between 2.7 and 3.4 million years ago to establish the derived *planitibia*, *neopicta*, and *cyrtoloma* subgroups, the basal members of which are found on Oahu. Three independent colonizations of the Maui Nui complex took place in each of these derived subgroups. Subsequent diversification on Maui Nui, either via vicariance or dispersal between different volcanic ranges, generated a diverse fauna of nine species in the past 2 million years. Additional work centering on population genetics within the Maui Nui *cyrtoloma*, *planitibia*, and *neopicta* groups should provide important insights into species formation in Hawaiian insects. A colonization of the Big Island by the *planitibia* subgroup took place about 600,000 years ago and led to the formation of two taxa, *D. heteroneura* and *D. silvestris*.

Acknowledgments

This paper is dedicated to the memory of the late D. Elmo Hardy, without whom none of this work could ever have been done. We thank the following individuals for helpful discussions and advice: Chelsea Specht (local clocks and divergence time estimations), Jonathan Price (Hawaiian biogeography and host plant evolution), and Hampton Carson (evolution of Hawaiian *Drosophila*). This study was supported by NSF Grants DEB 97-29191 to J.B. and DEB 01-29105 to P.O. and R.D.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.03.008.

References

Baker, R.H., DeSalle, R., 1997. Multiple sources of character information and the phylogeny of the Hawaiian Drosophilids. Syst. Biol. 46, 654–673.

Baker, R.H., Yu, X., DeSalle, R., 1998. Assessing the relative contributions of molecular and morphological characters in simultaneous analysis trees. Mol. Phylogenet. Evol. 9, 427–436.

Barrio, E., Ayala, F.J., 1997. Evolution of the *Drosophila obscura* species group inferred from the *Gpdh* and *Sod* genes. Mol. Phylogenet. Evol. 7, 1–15.

Benning, T.L., LaPointe, D., Atkinson, C.T., Vitousek, P.M., 2002. Interactions of climate change with biological invasions and land use in the Hawaiian Islands: modeling the fate of endemic birds using a geographic information system. Proc. Natl. Acad. Sci. USA 99 (22), 14246–14249.

Bremer, K., 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42, 795–803.

Bremer, K., 1992. Branch support and tree stability. Cladistics 10, 295–304.

Brower, A.V.Z., 1994. Phylogeny of the *Heliconius* butterflies inferred from protein comparisons. Proc. Natl. Acad. Sci. USA 82, 4753–4757.

Carson, H.L., 1971. Polytene chromosome relationships in Hawaiian species of *Drosophila*. V. Additions to the chromosomal phylogeny of the picture-winged species. Univ. Tex. Publ. 7103, 183–192.

Carson, H.L., 1992. Inversions in Hawaiian *Drosophila*. In: Krimbas, C.B., Powell, J.R. (Eds.), Drosophila Inversion Polymorphism. CRS Press, Boca Raton, FL, pp. 407–439.

Carson, H.L., 2004. Phylogeny and population genetics of Hawaiian Drosophilids: impact on evolutionary biology. Bishop Mus. Bull. Entomol. 10, 109–116.

Carson, H.L., Clague, D.A., 1995. Geology and biogeography of the Hawaiian Islands. In: Wagner, W.L., Funk, V.A. (Eds.), Hawaiian Biogeography, Evolution on a Hot Spot. Smithsonian Institution Press, Washington, DC, pp. 14–29.

Carson, H.L., Heed, W.B., 1986. Methods of collecting *Drosophila*. In: Ashburner, M., Carson, H.L., Thompson, J.N. (Eds.), The Genetics and Biology of *Drosophila*, vol. 3e. Academic Press, New York, pp. 1–28.

Carson, H.L., Kaneshiro, K.Y., 1976. *Drosophila* of Hawai'i: systematics and ecological genetics. Ann. Rev. Ecol. Syst. 7, 311–345.

Carson, H.L., Stalker, H.D., 1968. Polytene chromosome relationships in Hawaiian species of *Drosophila.* II. The *D. planitibia* subgroup. Univ. Tex. Publ. 6818, 355–366.

Carson, H.L., Templeton, A.R., 1984. Genetic revolutions in relation to speciation phenomena: the founding of new populations. Ann. Rev. Ecol. Syst. 15, 97–131.

Carson, H.L., Clayton, F.E., Stalker, H.D., 1967. Karyotypic stability and speciation in Hawaiian *Drosophila*. Proc. Natl. Acad. Sci. USA 57, 1280–1285.

Clague, D.A, Dalrymple, G.B., 1987. The Hawaiian Emperor volcanic chain. In: Decker, R.W., Wright, T.L., Stauffer, P.H. (Eds.), Volcanism in Hawai'i. U.S. Geological Survey Professional Paper 1350. U.S. Government Printing Office, Washington, DC, pp. 1–54.

Clayton, F.E., Carson, H.L., Sato, J.E., 1972. Polytene chromosome relationships in Hawaiian species of *Drosophila*. VI. Supplementary data on metaphases and gene sequences. Univ Tex. Publ. 7213, 163–178.

Craddock, E.M., 2000. Speciation processes in the adaptive radiation of Hawaiian plants and animals. Evol. Biol. 31, 1–53.

DeSalle, R., Templeton, A.R., 1988. Founder effects and the rate of mitochondrial DNA evolution in Hawaiian *Drosophila*. Evolution 42, 1076–1084.

DeSalle, R., Williams, A.K., George, M., 1993. Isolation and characterization of animal mitochondrial DNA. Methods Enzymol. 224, 176–204.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Testing the significance of incongruence. Cladistics 10, 315–319.

Farris, J.S., Albert, V., Kallersjo, M., Lipscomb, D., Kluge, A.G., 1996. Parsimony jackknifing outperforms neighbor-joining. Cladistics 12, 99–124.

Felsenstein, J.F., 1978. Cases in which parsimony or compatibility can be positively misleading. Syst. Zool. 27, 401–410.

Foote, D., Carson, H.L., 1995. *Drosophila* as monitors of change in Hawaiian ecosystems. In: LaRoe, E.T. (Ed.), Our Living Resources: A Report to the Nation on the Distribution, Abundance, and Health of U.S. Plants, Animals, and Ecosystems. United States Department of the Interior, National Biological Service 1995, pp. 368–372.

Gatesy, J.R., DeSalle, R., Wheeler, W., 1993. Alignment-ambiguous nucleotide sites and the exclusion of systematic data. Mol. Phylogenet. Evol. 2, 152–157.

Giddings, L.V., Templeton, A.R., 1983. Behavioral phylogenies and the direction of evolution. Science 220, 372–377.

Gillespie, R., 2004. Community assembly through adaptive radiation in Hawaiian spiders. Science 303, 356.

Hardy, D.E., Kaneshiro, K.Y., 1981. Drosophilidae of the Pacific Oceania. In: Ashburner, M., Carson, H.L., Thompson, J.J.N. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, New York, pp. 309–348.

Heed, W.B., 1968. Ecology of the Hawaiian Drosophilidae. Univ Tex. Publ. 6818, 388–419.

Ho, K.-F., 1994. Phylogeny of Hawaiian *Drosophila* inferred from yolk protein gene evolution. Ph.D thesis. New York University.

Ho, K.-F., Craddock, E.M., Piano, F., Kambysellis, M.P., 1996. Phylogenetic analysis of DNA length mutations in a repetitive region of the Hawaiian *Drosophila* yolk protein gene *Yp2*. J. Mol. Evol. 43, 116–124.

Hotchkiss, S., Juvik, J.O., 1999. A Late-Quaternary pollen record from Ka'au Crater, O'ahu, Hawai'i. Quat. Res. 52 (1), 115–128.

Hotchkiss, S., Vitousek, P.M., Chadwick, O.A., Price, J., 2000. Climate cycles, geomorphological change, and the interpretation of soil and ecosystem development. Ecosystems 3 (6), 522–533.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Jordan, S., Simon, C., Polhemus, D., 2003. Molecular systematics and adaptive radiation of Hawai'i's endemic damselfly genus *Megalagrion* (Odonata: Coenagrionidae). Syst. Biol. 52, 89–109.

Kambysellis, M.P., Ho, K.-F., Craddock, E.M., Piano, F., Parisi, F., Cohen, J., 1995. Pattern of ecological shifts in the diversification of Hawaiian *Drosophila* inferred from a molecular phylogeny. Curr. Biol. 5, 1129–1139.

Kaneshiro, K.Y., 1976a. A revision of generic concepts in the biosystematics of Hawaiian Drosophilidae. Proc. Hawaiian Entomol. Soc. 22, 255–278.

Kaneshiro, K.Y., 1976b. Ethological isolation and phylogeny in the *planitibia* subgroup of Hawaiian *Drosophila*. Evolution 30, 740–745.

Kaneshiro, K.Y., 1997. R.C.L. Perkins' legacy to evolutionary research on Hawaiian Drosophilidae, Diptera. Pacif. Sci. 51 (4), 450–461.

Kaneshiro, K.Y., Gillespie, R.G., Carson, H.L., 1995. Chromosomes and male genitalia of Hawaiian *Drosophila*: tools for interpreting phylogeny. In: Wagner, W.L., Funk, V.A. (Eds.), Hawaiian Biogeography Evolution on a Hot Spot. Smithsonian Institution Press, Washington, DC, pp. 57–71.

Kosakovsky-Pond, S., Muse, S., 2000. Hy-Phy. Available from: <http://peppercat.statgen.ncsu.edu/~hyphy>.

Kress, W.J., Specht, C.D., 2004. The evolutionary and biogeographic origin and diversification of the tropical monocot order Zingiberales. In: Columbus, J.T., Friar, E.A., Hamilton, C.W., Porter, J.M., Prince, L.M., Simpson, M.G. (Eds.), Monocots: Comparative Biology and Evolution. Rancho Santa Ana Botanic Garden, Claremont, CA.

O'Grady, P.M., 2002. Species to genera: phylogenetic inference in the Hawaiian Drosophilidae. In: DeSalle, R., Giribet, G., Wheeler, W. (Eds.), Molecular Systematics and Evolution: Theory and Practice. Birkhauser Verlag, Berlin, pp. 17–30.

O'Grady, P.M., Zilversmit, M., 2004. Phylogenetic relationships within the *haleakalae* species group inferred by molecular and morphological characters (Diptera: Drosophilidae). Bishop Mus. Bull. Entomol. 10, 117–134.

O'Grady, P.M., Clark, J.B., Kidwell, M.G., 1998. Phylogeny of the *Drosophila saltans* species group based on combined analysis of nuclear and mitochondrial DNA sequences. Mol. Biol. Evol. 15 (6), 656–664.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Price, J.P., Clague, D.A., 2002. How old is the Hawaiian biota. Geology and phylogeny suggest recent divergence. Proc. R. Soc. Lond. B 269, 2429–2435.

Price, J.P., Elliot-Fisk, D., 2004. Topographic history of the Maui Nui complex, Hawaii, and it's implications for biogeography. Pacif. Sci. 58, 27–45.

Russo, C.M.A., Takezaki, N., Nei, M., 1995. Molecular phylogeny and divergence times of Drosophilid species. Mol. Biol. Evol. 12, 391–404.

Sorenson, M.D., 1999. TreeRot, version 2. Boston University, Boston, MA.

Spieth, H.T., 1982. Behavioral biology and evolution of the Hawaiian picture-winged species group of *Drosophila*. Evol. Biol. 14, 351–437.

Swofford, D., 2004. Phylogenetic analysis using parsimony* version 4.ob4. Sinauer Associates, Sunderland, MA.

Thomas, R.H., Hunt, J.A., 1991. The molecular evolution of the alcohol dehydrogenase locus and the phylogeny of Hawaiian *Drosophila*. Mol. Biol. Evol. 8, 687–702.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1997. Clustal X. European Molecular Biology Laboratory, Heidelberg, Germany.

Wagner, W.L., Herbst, D.R., Sohmer, S.H., 1999. Manual of the Flowering Plants of Hawaii, second ed. University of Hawaii Press, Honolulu.

Wilkinson, G.S., Kahler, H., Baker, R.H., 1998. Evolution of female mating preference in stalk-eyed flies. Behav. Ecol. 9, 525–533.

Yang, Z., 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. CABIOS 13, 555–556. Available from: <http://abacus.gene.ucl.ac.uk/software/paml.html>.

Yoder, A.D., Yang, Z., 2000. Estimation of primate speciation dates using local molecular clocks. Mol. Biol. Evol. 17, 1081–1090.