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Phylogenetic Relationships Within the *Drosophila haleakalae* Species Group Inferred by Molecular and Morphological Characters (Diptera: Drosophilidae)

PATRICK M. O'GRADY[1, 2]
American Museum of Natural History, Division of Invertebrate Zoology,
Central Park West @ 79th Street, New York, NY 10024, USA; email: pogrady@uvm.edu

MARTINE ZILVERSMIT
Harvard University, Department of Organismic and Evolutionary Biology,
16 Divinity Avenue, Cambridge, MA 02138, USA

Abstract

The *Drosophila haleakalae* species group, the most basal lineage within the Hawaiian *Drosophila* lineage, consists of 54 described species placed in 5 subgroups. Previous taxonomic studies, initiated by Elmo Hardy, have provided an excellent groundwork on which to base further evolutionary studies. We present a phylogenetic hypothesis of the *Drosophila haleakalae* species group using a suite of morphological, behavioral, and molecular characters (including 5 newly developed nuclear gene regions) that is more resolved and better supported than any previous phylogeny of this group. We use our phylogeny to refine and revise the taxonomic relationships of species in the *haleakalae* species group.

Background

Soon after Elmo Hardy arrived at the University of Hawai'i in 1948, he began collaborating with E. C. Zimmerman to treat the endemic Hawaiian Diptera, an ambitious task that eventually resulted in 5 volumes in the Insects of Hawai'i series and an impressive series of additional publications (Evenhuis & Thompson, 2003). After starting work at UH Mānoa, Elmo began to accumulate data on the known Hawaiian Diptera (Hardy, 1952), as well as make collections of hundreds of new drosophilid species. The years 1950–1959 were filled with inter-island travel, often via boat or prop plane. For example, during April–August 1952, Elmo made collections on O'ahu, Maui, Moloka'i, Lāna'i, Hawai'i, and Kaua'i. He repeated this during the same period in 1953 and made similar expeditions in 1956, 1958 and 1959 (Hardy *et al.*, 2001). The largest and most diverse group that Elmo began to study in those early years was the Hawaiian Drosophilidae.

The early 1960s were an exciting time to study Hawaiian Drosophilidae. Not only did Elmo continue to collect and describe new species, he initiated the Hawaiian *Drosophila* Project with collaborators at the University of Texas and other institutions (Spieth, 1980, 1981). This joint NSF-NIH initiative began in 1963 with the goal to understand all aspects of the basic biology of the endemic Hawaiian Drosophilidae. Elmo's contributions to this project, along with the studies of Hamp Carson, Bill Heed, Herman Spieth, Ken Kaneshiro and others, have made the Hawaiian Drosophilidae one of the most powerful evolutionary model systems and the best documented example of adaptive radiation in nature (Craddock, 2000). Critical to the success of this work was the publication of Elmo's revision of the Hawaiian Drosophilidae, a work that included a treatment of all 400 drosophilids known from Hawai'i at that time, about 350 of which were newly described (Hardy, 1965). Subsequent publications extending into the late 1970s, many in collaboration with Ken Kaneshiro, added over 100 more species to this fauna.

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1. Present Address: University of Vermont, Department of Biology, 316 Marsh Life Sciences Building, Burlington, VT 05405
2. Author to whom correspondence should be addressed.

When Elmo retired from the University of Hawai'i in 1980, one major revision remained to completed, a treatment of the so-called "fungus feeder" species group. When Herman Spieth developed the now standard "mushroom tea" bait in the late 1970s, a whole new fauna of mycophagous *Drosophila* were discovered. Elmo, along with Kenneth Kaneshiro, began a revision of this new material during the 1970s. However, because of retirement and other concerns, their study was never published. Through the 1980s and 1990s various students and post-docs worked on the manuscript, but the general content of the work remained as Elmo and Ken had left it in 1980. Chica do Val and one of us (O'Grady) began to revise this work during the summer of 1999 and published it (Hardy *et al.*, 2001), renaming the group *haleakalae*, after a name first used by Elmo Hardy (1965).

Our work on the *haleakalae* revision has in turn stimulated additional research. For example, I was very interested in understanding the phylogenetic relationships among species in this enigmatic group and thought that DNA sequences might be able to resolve some issues that morphology alone could not. Martine Zilversmit and I present the results of this research here. It is a pleasure to dedicate this paper to the memory Dr. Elmo Hardy, a man whose long career and diligent work has had a significant impact not only on the Hawaiian Drosophilidae, but many other groups of Diptera (*e.g.,* Tephritidae, Bibionidae, Pipunculidae, Dolichopodidae) as well.

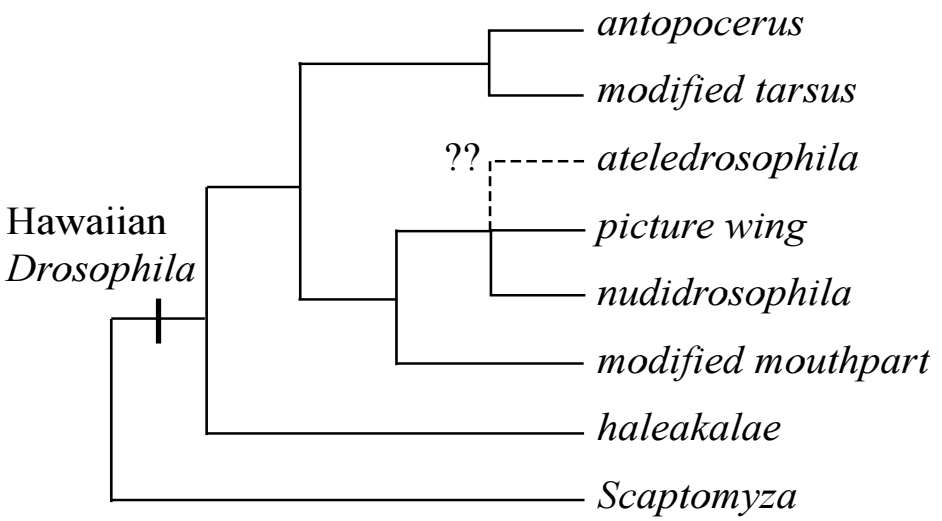
Introduction

The Hawaiian Drosophilidae consists of two major lineages, Hawaiian *Drosophila* and the genus *Scaptomyza* (Fig. 1; Bonacum, 2001; O'Grady, 2002; O'Grady *et al.*, 2003; Remsen and O'Grady, 2002). Within the Hawaiian *Drosophila* lineage, there are currently 7 recognized species groups (*antopocerus*, *ateledrosophila*, *haleakalae*, modified mouthpart, modified tarsus, *nudidrosophila*, picture wing). The *haleakalae* species group is the most basal and contains a total of 54 species, all of which are endemic to the Hawaiian Islands (Hardy *et al*., 2001). Although this group was first formally proposed and named by Hardy *et al*. (2001) it has been known by a variety of names over the past 40 years, including "fungus feeders", "rimmed labellum", and "white (or light) tipped scutellum group" (e.g., Heed, 1968; Spieth, 1966; Throckmorton, 1966). Based on morphological characters, Hardy *et al.* (2001) divided this group into 6 subgroups: *anthrax*, *cilifemorata*, *haleakalae*, *luteola*, *polita*, and *scitula*. These characters, however, consisted of only a few "key characteristics" that were used to separate species and were never analyzed using cladistic methods. The potential suite of morphological characters available to examine relationships in this group was not yet comprehensively surveyed.

Throckmorton (1966) listed a number of synapomorphies for the *haleakalae* group, including male genitalia lacking anal sclerite, short filaments on eggs, and females with weakly sclerotized, non-telescoping and non-functional spermathecae. Spieth (1966) also observed that all members of this group lack the elaborate courtship displays seen in the other major lineages of Hawaiian *Drosophila*. Several molecular studies have tested the monophyly of this group in a maximum parsimony framework (Kambysellis *et al.*, 1995; Baker & DeSalle, 1997; Bonacum, 2001), but none sampled extensively within the *haleakalae* species group.

Bonacum (2001), who used about 3.3 kb from four loci (16S, COI/COII, *Adh, Gpdh*) to examine phylogenetic relationships among the major Hawaiian drosophilid lineages sampled more extensively within the *haleakalae* group than any other previous study. He included 13 *haleakalae* group species in his study (Fig. 2). Only 3 nodes showed significant bootstrap support: (A) *D. nigella-D. fungiperda*, (B) *D. nigella-D. fungiperda-D. nigra* and (C) *D. inciliata-D. longiperda*. Nodes A (*fungiperda* complex) and C (*venusta* cluster) correspond well with the taxonomy proposed by Hardy *et al*. (2001). Node B suggests that the *cilifemorata* cluster may not be monophyletic due to the placement of *D. nigra* as the sister of the *fungiperda* complex. This grouping makes sense from a morphological standpoint, however, because all 3 taxa lack a rimmed labellum.

We analyzed a total of 18 ingroup and 3 outgroup taxa in order to test the monophyly of the *haleakalae* species group and its component subgroups using a combination of characters. A total of 87 morphological and behavioral characters were scored and analyzed using maximum parsimo-



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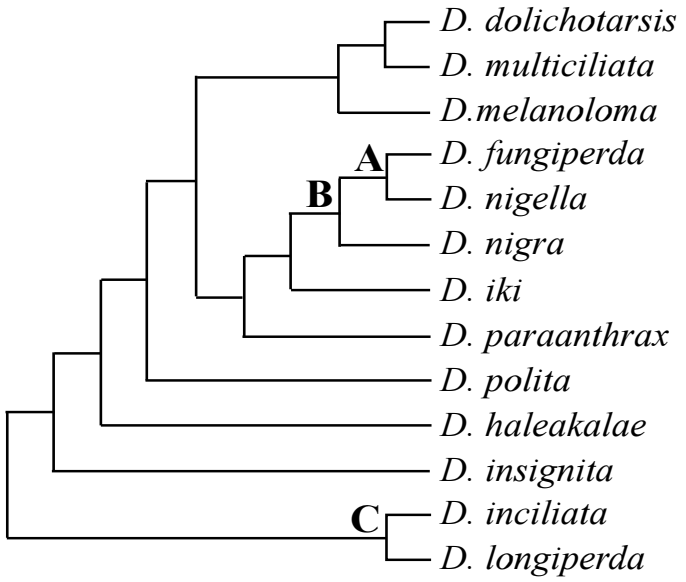

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Figure 1. Phylogenetic relationships among the major lineages of the Hawaiian Drosophilidae. **Figure 2.** Phylogenetic relationships within the *haleakalae* species group, after Bonacum (2001). Letters at the nodes indicate those relationships which were strongly supported by bootstrap proportions.

ny. The molecular matrix, containing over 5000 characters from eight rapidly evolving gene regions (COII, *sia, glass, l(2)not-1*, *Marf*, *Rpt4*, ITS-1, *snf*) and a presence-absence insertion-deletion (indel) matrix was also analyzed using maximum parsimony. Maximum likelihood was employed to further analyze the molecular loci, both individually and in combination with one another. Finally, a data set consisting of both molecular, morphological, behavioral, and indel characters was analyzed using maximum parsimony.

Our results suggest strong support for the monophyly of the *haleakalae* species group. While a number of previously proposed (Hardy *et al.*, 2001) clades are supported in the current study, several novel relationships are also observed. While the individual molecular and morphological analyses are largely unresolved, much stronger support is seen in the combined analyses. The approach taken in the current study highlights the benefit of using all available sources of character information including molecular, morphological, and ecological when inferring phylogenetic relationships.

Materials and Methods

Taxon Sampling

Taxa and localities sampled are listed in Table 1. Ingroup taxa were selected in order to sample from each major lineage within the *haleakalae* species group. Outgroup taxa were selected from 3 other Hawaiian *Drosophila* species groups: the picture wing (*D. crucigera*), modified mouthpart (*D. mimica*), and modified tarsus (*D. petalopeza* for the COII partition, *D. waddingtoni* for the *glass* partition, and *D. quasiexpansa* for all other data partitions).

Morphological and Behavioral Characters

A total of 87 morphological and behavioral characters were scored. The morphological characters were from external adult structures (this study), as well as internal morphology and immature forms (after Throckmorton, 1966). External adult structures were scored after surveying the literature (Hardy, 1965; Hardy *et al.*, 2001) and examining at least 10 individuals. Behavioral characters were scored after Spieth (1966).

Template Selection

With the exception of COII and ITS, the 8 loci we used were selected based on a previous study designed to examine phylogenetic relationships within the family Drosophilidae (Zilversmit *et al.*, 2002b). All sequences in the present study were chosen based on (1) the ease of amplification and sequencing and (2) because they appeared to be accumulating variation at a rate that would provide resolution at the species-level (based on the number of parsimony informative characters for each partition found in the pilot study).

Additional characters, generated by scoring indel events in the non-coding region of the *Marf* locus, were also analyzed. This region yielded a total of 68 characters, 24 of which were parsimony informative. All indel characters were considered discrete and were scored as either present or absent. The majority of indels were small (4–6 base pairs) and were present (or absent) in only a few taxa. Overlapping gapped regions were considered individual, discrete characters, rather than continuous varieties of the same character (Simmons & Ochoterena, 2000; Simmons *et al*., 2001).

DNA Isolation and PCR Amplification

In most cases, DNA was prepared from multiple flies (3–5). *Drosophila dolichotarsis* DNA was generated using a single fly. Flies were macerated using a micro pestle in a 1.5 ml PCR tube with buffer provided by the DNeasy Tissue Kit and DNA was isolated using the standard protocol supplied in the manufacturer's instructions (Qiagen). Loci of interest (above) were PCR-amplified using primers described in O'Grady (1999) and Bonacum (2001) employing the protocols of Zilversmit *et al.* (2002a). All sequences have been submitted to GenBank under accession numbers AY343526-AY343539 and AY348178-AY3481290. Several taxa are missing sequences for *glass*, *snf* and *Rpt4* as they were unable to be amplified from these templates.

Table 1. Taxonomy and Collection Information of Species Sampled

Group	Subgroup	Species	Collection Locality[1]
haleakalae	*anthrax*	*melanoloma*	MOLOKA'I: Pu'u Kolekole, 19—21.iii.1999, OG58.6, PMO&JBS
		multiciliata	HAWAI'I: Kipuka #9, Saddle Road, 5100 ft., 12.i.1988, Y37, RD
	cilifemorata	*longiperda*	HAWAI'I: Ola'a Forest, Volcanoes National Park, 14—15.iii.1989, Y57, RD,JS&KS
		dolichotarsis	MAUI: Waikamoi Forest Preserve, 7—8.vi.1997, Z35, MPK,CM&SLM
		iki	MAUI: Waikamoi Forest Preserve, JB&MPK
		nigra	MAUI: Waikamoi Forest Preserve, JB&MPK
		insignita	O'AHU: Makua Valley, 20.ii.2000, OG86.1, PMO,JBS&SLM
	haleakalae	*ochropleura*	HAWAI'I: Greenwell Ranch, Pauahi, Kona, 18.ii.2000, OG84.1, PMO&JBS
		fungiperda	HAWAI'I: Kaloko Mauka, North Kona, 3.vii.1998, OG38.5, PMO&SLM
		nigella	MAUI: Waikamoi Forest Preserve, JB&MPK
		haleakalae	MAUI: Paliku Cabin, Haleakala, 6400 ft., 3.viii.1988, Y50, KYK,RD,RA&WDP
	polita	*bipolita*	O'AHU: Ekahanui Gulch, Waianae Mountains, 10.ii.2000, OG75.5, PMO,MPK&SLM
		canipolita	O'AHU: Makua Valley, 20.ii.2000, OG86.2, PMO,JBS&SLM
		paraanthrax	KAUA'I: Pihea Trail, Na Pali Kona Forest Preserve, 25.ii.2000, OG89.2, PMO&JBS
		polita	HAWAI'I: Greenwell Ranch, 12—14.iii.1989, Y56, KYK,RD,JSY,KSY&RDS
	scitula	*fulgida*	KAUA'I: Honapu Ditch Trail, Puu Ka Pele Forest Preserve, 24.ii.2000, OG87.9, PMO&JBS
		melanosoma	KAUA'I: Honapu Ditch Trail, Puu Ka Pele Forest Preserve, 24.ii.2000, OG87.8, PMO&JBS
		scitula	KAUA'I: Honapu Ditch Trail, Puu Ka Pele Forest Preserve, 24.ii.2000, OG87.E, PMO&JBS
mod. mouthpart		*mimica*	HAWAI'I: Hawai'i Volcanoes National Park, JB&MPK
modified tarsus		*petalopeza*	MAUI: Upper Waikamoi Forest Preserve, 6.vii.1998, OG41.2, PMO&SLM
		waddingtoni	MAUI: Heed Trail, Waikamoi Forest Preserve, 2.vi.1999, OG71.A, PMO
		quasiexpansa	MAUI: Upper Waikamoi Forest Preserve, 6.vii.1998, OG41.E, PMO&SLM
picture wing		*crucigera*	O'AHU: RD&KYK Collection, W41N3

1. Collector Abbreviations: CM = Cam Muir; JB = James Bonacum; JBS = Julian B. Stark; JSY = Jong Yoon; KSY = Kay Yoon; KYK = Kenneth Y. Kaneshiro; MPK = Michael P. Kambysellis; PMO = P. M. O'Grady; RA = Ross Antonson; RD = Rob DeSalle; SLM = Steven L. Montgomery; WDP = William D. Perreira

Table 2. Summary of Maximum Parsimony Analyses.

Partition	# Characters	#PICs[1]	%PICs	#MPTs[2]	# Steps	CI[3]	RI[4]
All Data	5121	508	—	2	2295	0.723	0.501
molecular	4966	431	85	3	1999	0.75	0.513
nuclear	4278	322	63	5	1520	0.824	0.599
COII (mt)	688	109	21	3	459	0.538	0.408
sia	462	14	3	50,000+[6]	106	0.925	0.75
glass[5]	613	23	5	24	95	0.937	0.842
ITS-1	661	81	16	612	327	0.838	0.685
l(2)not-1	638	83	16	878	228	0.811	0.684
snf[5]	467	26	5	6	178	0.933	0.714
Marf	954	113	22	16	467	0.857	0.73
Rpt4[7]	483	21	4	nd	nd	nd	nd
indels	68	24	5	27	71	0.915	0.846
morphology	87	53	10	36	193	0.487	0.533

1. Parsimony Informative Characters.
2. Most Parsimonious Trees.
3. Consistency Index.
4. Retention Index.
5. Sequences available from only a subset of taxa. Searches with cg3455 not attempted, only four taxa determined.
6. Search could not be completed due to lack of memory (too many equally parsimonious trees). Maxtrees set to 50,000 for *sia* search.
7. Search not done because only a few taxa amplified for this locus.

Sequence Editing and Phylogenetic Analysis

All sequences were edited in Sequencher 4.0 (Gene Codes Corp.) and exported into NEXUS formatted files (Maddison *et al.*, 1999). Alignment for protein coding sequences was trivial and was done manually. Non-coding regions were also aligned manually using MacClade (Maddison & Maddison, 2000). Alignments are available from the authors by request.

Phylogenetic analyses, using both maximum parsimony (MP) and maximum likelihood (ML) algorithms, were done in PAUP* 4.0b10 (Swofford, 2003). In addition to analyzing all data in a simultaneous analysis (Nixon & Carpenter, 1996), we also partitioned the data as follows: morphology and behavior alone, all molecular characters, nuclear loci, mitochondrial loci, and individual analysis of all partitions (COII, ITS, *Marf*, *sia, snf, glass, l(2)not*, indels). Settings for MP analyses were as follows: search type = heuristic, addition sequences = random, number of replicates = 200, branch swapping = TBR. Support at each node was assessed using bootstrap proportions (BP; Felsenstein, 1985, 1988) and Jackknife (JK; Farris *et al.*, 1996) with 200 bootstrap or jackknife replicates (other settings as above). Uninformative characters were excluded for bootstrap replicates. Jackknife was done using a 37% deletion with the emulate Jac resampling option selected. Decay indices (Bremer, 1988) and partitioned branch support (PBS; Baker & DeSalle, 1997) were calculated using TreeRot (Sorenson, 1999).

Modeltest (Version 3.06; Posada & 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, 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).

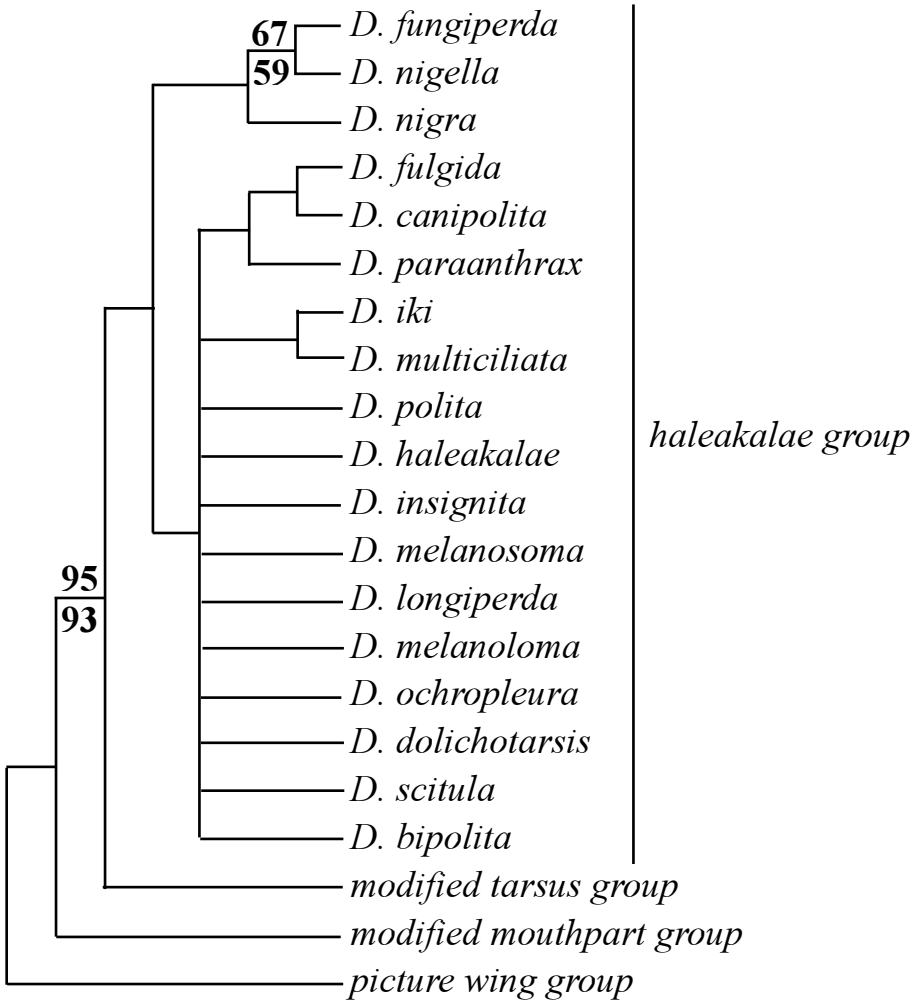

Figure 3. Morphological hypothesis of relationships within the *haleakalae* species group. Phylogeny shown is a strict consensus of 36 most parsimonious trees of 193 steps (refer to Table 1 for more information). Bootstrap proportions are above the nodes and jackknife values are below.

Divergence Time Estimation

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 & Crandall, 1998) so we will use a version of the local clock (Yoder & Yang, 2001). Pairwise relative rate tests (outgroup = picture wing group, model = GTR) were performed using HYPHY, version 0.95beta (Kosakovsky-Pond & Muse, 2000) to determine rate classes for various branches. Pairwise comparisons which 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 (Yoder & Yang, 2000). Divergence times were estimated in PAML, version 3.13 (Yang, 1997) using three calibration points (node 5) *D. nigella-D.*

fungiperda, (node 1) *D. ochropleura-D. haleakalae*, and (node 10) *D. bipolita-D. canipolita-D. insignita*. Nodes 1 and 5 were set to 0.5 and 1.9 million years (MY), the geological age of the Big Island and Maui, respectively. This gives an upper and a lower bound of divergence estimates. Node 10 was set to 3.7 MY, the age of O'ahu, the oldest island with all three species present. Two different local clocks were used: TREE 1, where one rate class was assigned to *D. fungiperda*, another to *D. fulgida* and a third to all other branches and TREE 2 where one rate class was given to the outgroup, another to the short internodes (Fig. 5), and a third to all other branches.

The calibration point at node 5 is problematic as *D. fungiperda* is involved in many significantly heterogeneous pairwise relative rate tests (data not shown). In fact, *D. fungiperda* and the closely related *D. fulgida* together account for over 2/3 of the significant pairwise relative rate test results. An attempt to correct for this heterogeneity (tree 1, above) still yielded divergence time estimates that were very recent, given the distribution of taxa. For this reason, only points 2 and 3 were used. Values from each estimate were averaged (after Jordan *et al.*, 2003) and are presented in Fig. 6.

Results

Morphology and Behavior

Maximum parsimony analysis of the morphology and behavior data matrix recovered 36 equally parsimonious trees of 193 steps (Table 2). The strict consensus of these trees is largely unresolved (Fig. 3), although there is support for a *nigella-fungiperda* clade (BP = 67, JK = 59) and the monophyly of the *haleakalae* group as a whole (BP = 95; JK = 93). Several other relationships are seen in the strict consensus (Fig. 3), but are not supported in either the bootstrap or jackknife analyses. It is clear that, while over 50% of the characters in this partition are parsimony informative, these aren't sufficient to provide support for any but the most robust nodes. Sampling additional morphological characters might be possible but, because members of the *haleakalae* group are quite homogeneous with respect to external morphology, this will require extensive scanning electron microscopy and dissection of internal structures.

Individual Analyses of Molecular Partitions

Individual partitions analyzed using MP (Table 2) and ML (Table 3) displayed varying levels of resolution based on a variety of factors (number of parsimony informative characters, signal to noise ratio, inferred base composition and rate matrices, etc.). Not surprisingly, these smaller partitioned data sets were not as well resolved or supported as the larger combined partitions. However, several relationships were common to both the combined and multiple individual partitions and likely reflect cases of strong support in the data. Rather than present each individual phylogeny, we summarize recurring clades, (Table 4; Figs. 4, 5). Of the 14 nodes present in the combined MP search, 10 were recovered in at least 2 individual analyses and over half (6) were supported in 3 or more of the individual searches (Table 4).

Several of these nodes correspond well with the taxonomic groups proposed by Hardy *et al.* (2001). For example, the monophyly of the *haleakalae* group (Fig. 4; node 17) is supported in all of the individual MP analyses, regardless of the partition examined. The *fungiperda* complex (Fig. 4; node 5), is supported in five individual analyses. Interestingly, one other relationship (Fig. 4; node 11) was also found in 5 individual partitions, but did not exactly correspond with any taxonomic group proposed by Hardy *et al*. (Table 5). In this case, a modified version of the *polita* subgroup, including *D. insignita*, should be erected to reflect the recent phylogenetic results (see below).

Individual MP topologies were highly congruent with both individual and combined ML trees (Table 4). Although it is not possible to partition support on the ML trees, the presence of several key nodes (i.e., *haleakalae* group, *fungiperda* complex) in multiple individual ML searches lends support to these relationships (Table 4). Interestingly, those clades present in individual ML analyses, but not seen in corresponding MP trees, were typically supported by a positive partitioned branch support value in combined analyses (Table 4).

Table 3. Summary of Maximum Likelihood Analyses.

Partition	Model[1]	-ln L	G[2]	I[3]	Base Frequencies	Rate Matrix
molecular	GTR+I+G	17878.1191	0.7654	0.3513	A = 0.2776 C = 0.2278 G = 0.2115 T = 0.2831	A-C = 1.0391 A-G = 2.2446 A-T = 1.5406 C-G = 0.9470 C-T = 3.0925 G-T = 1.0000
completed	GTR+I+G	13795.97434	0.7819	0.3521	A = 0.2857 C = 0.2088 G = 0.1962 T = 0.3093	A-C = 1.1412 A-G = 2.2862 A-T = 1.4604 C-G = 1.1112 C-T = 3.3180 G-T = 1.0000
nuclear	HKY+G	14570.5479	0.4135	0	A = 0.2778 C = 0.2303 G = 0.2147 T = 0.2773	TRatio = 0.8687
COII (mt)	GTR+I+G	3344.0908	0.4413	0.4510	A = 0.3278 C = 0.1491 G = 0.1170 T = 0.4061	A-C = 1.0000 A-G = 6.3467 A-T = 1.0000 C-G = 1.0000 C-T = 11.5958 G-T = 1.0000
glass	HKY+G	1474.9418	0.4413	0	A = 0.2417 C = 0.3086 G = 0.2289 T = 0.2208	TRatio = 1.3424
ITS-1	HKY+G	2698.9194	0.7189	0	A = 0.3907 C = 0.1292 G = 0.1237 T = 0.3563	TRatio = 0.5673
l(2)not-1	K80+G	2218.4741	0.3157	0	equal	TRatio = 1.7308
snf	HKY+G	1445.3038	0.6670	0	A = 0.3324 C = 0.2049 G = 0.2332 T = 0.2295	TRatio = 1.0547
Marf	F81+G	3853.4568	0.703	0	A = 0.2671 C = 0.2209 G = 0.2103 T = 0.3017	na

1Models of evolution used include: the Felsenstein 1981 model (F81) which allows for unequal base frequencies, 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.

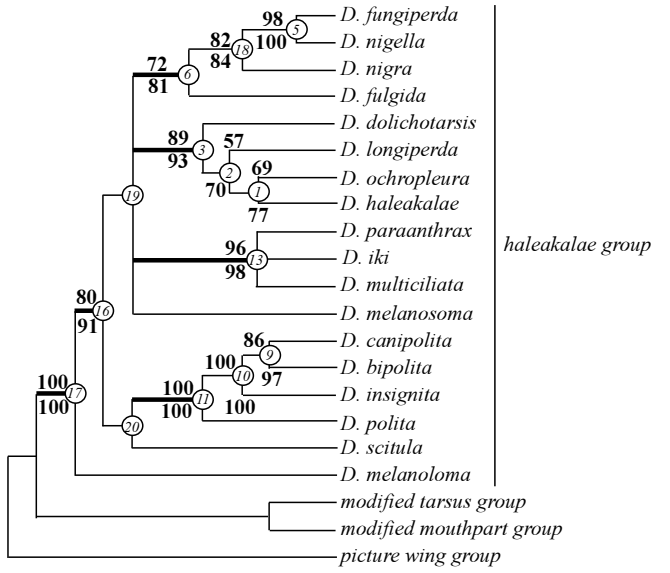

Figure 4. Phylogenetic relationships within the *haleakalae* species group based on maximum parsimony analysis of all molecular and morphological data. Bootstrap proportions are shown above each node, jackknife values are below. Small italic numbers at each internode refer to various clades (see Results; Table 4).

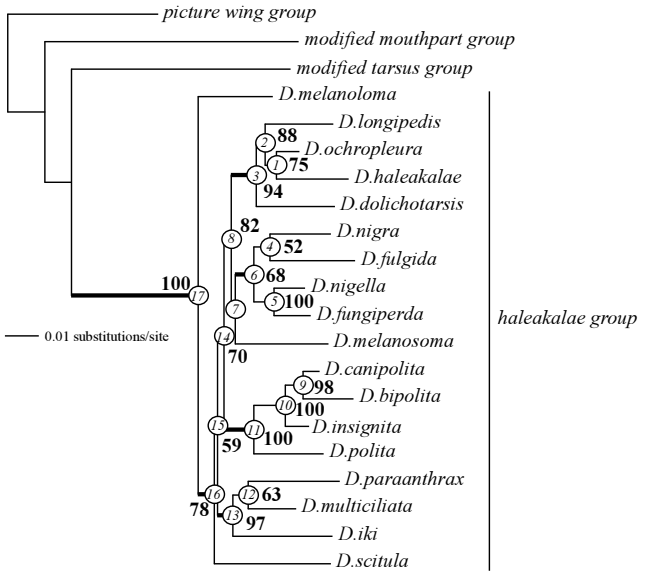

Figure 5. Phylogenetic relationships within the *haleakalae* species group based on maximum likelihood analysis of all molecular loci. Bootstrap proportions are shown above each node. Small, circled italic numbers at each internode refer to various clades (see Results; Table 4).

Combined Analyses

Tree topologies from combined MP and ML analyses are very similar to one another (Figs. 4, 5). Of the fourteen nodes present on the combined MP analysis, 11 were also observed in the combined ML phylogeny. There are only 3 cases where a relationship is supported with MP but not with ML, and only 6 cases where the converse is true (Table 4). These latter differences are due mainly to the differences in support and resolution between the MP tree and the fully resolved ML phylogeny. If one were to exclude those nodes lacking greater than 63% BP in either combined analysis (Figs. 4, 5; nodes 4, 7, 8, 12, 15, 19, 20), only 2 nodes actually differ. Of these, one (Fig. 4; node 18) is observed in partitions for which ML analyses were not tractable (Fig. 3; morphology). The remaining relationship (Fig. 5; node 14) is observed in some individual analyses, suggesting only minimal support (BP = 70%) for these clades in the combined ML analyses. We discuss the ramifications of the current analyses to the taxonomic and phylogenetic relationships of the *haleakalae* species group below.

Phylogenetic Relationships

The *haleakalae* species group is difficult to work with taxonomically because, unlike the related Hawaiian *Drosophila* species groups, very few characters exist that define species or aggregates of species (*e.g.,* the *split tarsus* subgroup of the *modified tarsus* species group; Hardy & Kaneshiro(1979)). This is probably due to the fact that, like the closely related genus *Scaptomyza*, these species have relatively simple mating behaviors (Spieth, 1966). This tends to reduce the role of sexual selection in the generation of sexual dimorphism and other morphological differentiation. The placement of the *haleakalae* group as basal within the Hawaiian *Drosophila* (Throckmorton, 1966), close to the divergence of the genus *Scaptomyza*, suggests that extensive sexual dimorphism observed in more derived Hawaiian drosophilid taxa evolved after the divergence of the *haleakalae* group. Based on this phylogenetic position, it is clear that widespread sexual dimorphism, and the fascinating mating behaviors which characterize the majority of Hawaiian *Drosophila*, evolved after the divergence of the *haleakalae* species group.

The taxonomic framework proposed by Hardy *et al*. (2001) was based on only a few key morphological characteristics and was intended to be a working hypothesis of relationships within this difficult to characterize group, rather than a formal phylogenetic hypothesis. The work of Bonacum (2001) improved on this framework, generating phylogenetic support for the *fungiperda* complex (Fig. 2, A: *D. fungiperda* & *D. nigella*) and *venusta* cluster (Fig. 2, C: *D. inciliata* & *D. longiperda*). His results also call into question the monophyly of the *cilifemorata* subgroup. This heterogeneous clade contains a number of species (Table 1), including *D. nigra*, which is strongly supported as the sister of the *fungiperda* complex (of the *haleakalae* subgroup) rather than a member of the *cilifemorata* subgroup (Figure 2, B). The present study further expands on the previous work by sampling multiple individuals from 4 of the 5 proposed subgroups and including 87 morphological and behavioral characters and over 5000 base pairs of rapidly evolving molecular characters. This study is able to provide significant support for 14 of the 17 nodes in the ingroup (compared to 3 of 12 in the previous phylogenetic work (Bonacum, 2001)). As such, we are now able to propose a phylogenetic framework of relationships within the *haleakalae* species group. Table 5 summarizes the changes we propose.

In spite of strong support for the monophyly of the *haleakalae* species group (node 17; Fig. 4, BP & JK = 100, DI = 66; Fig. 5, BP = 100), it is clear that 2 of the subgroups proposed in Hardy *et al.* (2001), *scitula* and *cilifemorata*, are polyphyletic. Some of the taxa initially placed in these subgroups clearly belong to other clades and will be removed. For example, *D. insignita* (*cilifemorata* subgroup, *insignita* complex) is nested within a clade of species placed in the *polita* subgroup (node 11; Fig. 4, BP & JK = 100, DI = 13; Fig. 5, BP = 100). We propose that *D. insignita* be removed from the *cilifemorata* subgroup and be placed in the *polita* subgroup (Table 5). Two closely related taxa, *D. chicae* and *D. curtitarsis*, were not sampled in our study but have also been transferred to the *polita* subgroup, although their exact placement will hinge upon future phylogenetic work. Likewise, it is clear that *D. nigra* (*cilifemorata* subgroup, *cilifemorata* cluster) should be considered

Table 4. Comparison of Individual and Combined Analyses.

Node[1]	MP&ML[2]	DI[3]	Individual Search[4]	Positive PBS[5]	Negative PBS[6]
1	++	5	**COII** [4], **ITS** [0]	l(2)not [4], snf [2]	indels [-1], Marf [-4]
2	++	2	**Marf** [2]	l(2)not [5], snf [2]	COII [-3], ITS [-2], morph [-2]
3	++	7	**ITS** [5]	COII [1], sia [1], l(2)not [2], snf [2]	glass [-1], Marf [-3]
4	ML	na	**l(2)not**		
5	++	12	**COII** [9], **ITS** [2], ***l(2)not*** [2], ***Marf*** [0], morph [2]	snf [2]	sia [-1], glass [-4]
6	++	4	***l(2)not*** [6], snf [2]		COII [-2], Marf [-2]
7	ML	na			
8	ML	na	**Marf**		
9	++	5	***Marf*** [7]	morph [2]	ITS [-2], indels [-2]
10	++	18	COII [9], glass [0], **ITS** [2], ***Marf*** [2], indels [5]		l(2)not [-1]
11	++	13	**ITS** [7], ***Marf*** [2], indels [2]	COII [5], snf [1]	l(2)not [-4]
12	ML	na	COII, ITS		
13	++	7	glass [0], **ITS** [2], Marf [6]	l[2]not [2], snf [2], morph [4]	COII [-8], indels [-1]
14	ML	na	**ITS**		
15	ML	na	**ITS**		
16	++	8	***Marf*** [4], morph [0], indels [1]	sia [3], **ITS** [4]	COII [–2], glass [-1], snf [-1]
17	++	66	COII [0], sia [2], glass [0], **ITS** [11], ***l(2)not*** [14], ***Marf*** [27], indels [4], morph [9], ***snf*** [-1]		
18	MP	3	snf [1], morph [3], **ITS**	sia [1], Marf [1]	COII [-2], l(2)not [-1]
19	MP	2	l(2)not [1], snf [2],	Marf [1]	COII [-2]
20	MP	1	l(2)not [0]	sia [2], ITS [2], snf [3]	COII [-2], Marf [-1], indels [-1], morph [-2]

1. Node recovered in either combined MP or ML analyses (see Figs. 4, 5).
2. Relationship is present in combined MP, ML, or both (++) analyses.
3. Decay index (DI) in combined MP tree. Nodes only found in ML analyses have no DI.
4. Individual partition search in which specified node is present, along with partitioned branch support from the combined MP analysis in [ ]. Taxa in bold also appear in individual ML analyses. Bold, underlined partitions are present in individual ML and combined ML analysis but not present in MP searches.
5. Partitions contributing positive support to relationship in combined MP analysis, but not supporting node in individual analyses.
6. Partitions contributing negative support to relationship in combined MP analysis, but not supporting node in individual analyses.

a member of the *fungiperda* complex (node 18; Fig. 4, BP = 82, JK = 84, DI = 3; node 6; Fig. 5, BP = 68). All 3 of these species (*D. nigra, D. fungiperda, D. nigella*) lack a rimmed labellum and it is clear based on both morphological and molecular characters that they form a clade (Figs. 3–5).

Drosophila iki, another species initially placed in the *cilifemorata* cluster, should be removed to the newly erected *iki* complex within the *haleakalae* subgroup (Table 5) with 2 other species, *D. multiciliata* and *D. paraanthrax* (node 13; Fig. 4; BP = 96, JK = 98, DI = 7; Fig. 5, BP = 97). It is clear that the remaining taxa in the *cilifemorata* subgroup, *D. dolichotarsis* and *D. longiperda*, are closely related to the *haleakalae* subgroup (node 3; Fig. 4, BP = 89, JK = 93, DI = 7; Fig. 5, BP = 94). We suggest placing the remaining complexes and clusters of the *cilifemorata* subgroup into the *haleakalae* subgroup until additional taxon sampling can be undertaken (Table 5). This placement, while greatly increasing the size of the *haleakalae* subgroup, reflects our current understanding of phylogenetic relationships within the *haleakalae* species group.

The transfer of *D. multiciliata* to the *iki* cluster means that *D. melanosoma*, the only remaining member of the *anthrax* subgroup sampled (Table 5) is basal to all the remaining *haleakalae* group species (node 16; Fig. 4, BP = 80, JK = 91, DI = 8; Fig. 5, BP = 78). This relationship was suggested by Hardy *et al.* (2001) but should be tested by sampling additional taxa. The transfer of *D. paraanthrax* to the *iki* cluster, along with the transfer of *D. insignita* to the *polita* complex (Table 5), renders the *polita* complex monophyletic (node 11; Figs. 4, 5).

The 3 members of the *scitula* subgroup that we sampled in this study are also not monophyletic. One, *D. fulgida*, should be placed in the *fungiperda* complex of the *haleakalae* subgroup based on both the MP (Fig. 4; BP = 72, JK = 81, DI = 4) and ML (Fig. 5; BP = 68) analyses. The placement of *D. melanosoma* and *D. scitula* is somewhat more problematic and represents 2 of the more poorly supported clades in either phylogeny (Figs. 4, 5). *Drosophila melanosoma* has some affinities with the *haleakalae* subgroup, although it is not firmly allied with any one subgroup. *Drosophila scitula* is either basal to the *polita* subgroup (Fig. 4) or basal to all but the *anthrax* subgroup (Fig. 5). These 2 species, along with the remaining members of the *scitula* subgroup, *D. setositibia* and *D. subopaca*, should remain as unplaced in the *haleakalae* species group until additional work is done to more firmly assess their phylogenetic location (Table 5).

Divergence Times and Biogeographic Patterns

We are interested in estimating the divergence times of the major lineages within the *haleakalae* species group, as well as the age of the group as a whole in order to better understand the evolutionary dynamics that have shaped this group and other clades of Hawaiian *Drosophila*. Three calibration points (see Materials and Methods) were used to estimate divergence dates with a local molecular clock (Yoder & Yang, 2000). Because of heterogeneity within the *fungiperda* complex (above), we discarded this calibration point and used the mean divergence between *D. haleakalae-D. ochropleura* (node 1; 0.5–1.9 MY) and *D. bipolita-D. canipolita-D. insignita* (node 10; 3.7 MY). Ranges shown in Figure 6 are means from the local clocks specified by the TREE 1 and TREE 2 constraints.

Based on our estimates, the *haleakalae* species group diverged from the picture wing species group 20–21 MY ago. This is in agreement with the age estimates for the origin of the Hawaiian *Drosophila* at 26 MY (DeSalle, 1992; Russo *et al.*, 1995) and the placement of the *haleakalae* group as basal in the Hawaiian *Drosophila* radiation (Baker & DeSalle, 1997; Bonacum, 2001; O'Grady, 2002; Throckmorton, 1966). However, at that point in time little high elevation rainforest, the habitat required by all Hawaiian *Drosophila*, existed (Price & Clague, 2002). It is interesting to note that the major diversification of the *haleakalae* species group did not occur until about 10 MY ago (Fig. 6), when more suitable habitat was present on Gardner Pinnacles, La Perouse, and Necker Islands (Price & Clague, 2002). This pattern might suggest either evolutionary stasis or extensive extinction at the base of this lineage 20–10 MY ago. It might also be that sampling additional taxa within the basal *anthrax* subgroup could move the age of this group back to perhaps 15 MY, when large amounts of rainforest habitat was present on what is now Gardner Pinnacles (Price & Clague, 2002).

The ages of the major, well supported lineages within the *haleakalae* species group are all very similar, between 4.0 and 6.3 MY (Fig. 6). These groups are all quite recent, on the order of the age

Table 5. Revision of phylogenetic relationships in the *Drosophila haleakalae* species group.

ID	Taxon	Hardy *et al*., 2001	Current Study
I.	*anthrax* subgroup	*anthrax, demipolita, fascigera, fuscifrons, hemianthrax,* ***melanoloma, multiciliata****, nigropolita, retrusa, seorsa*	*anthrax, demipolita, fascigera, fuscifrons, hemianthrax,* ***melanoloma****, nigropolita, retrusa, seorsa*
II.	*cilifemorata* subgroup	*inciliata,* ***longiperda****, tanytarsis, venusta, cilifemorata,* ***dolichotarsis****, flaviceps,* ***iki, nigra****, stenoptera, swezeyi, denotata, sabroskyi, insignita*	This subgroup is not monophyletic. While it was possible to removed some one taxon (*insignita*) and place it in the *polita* subgroup, the majority of species here are actually closely related to several different *haleakalae* subgroup clades and will be considered as part of the *haleakalae* subgroup.
III.	*haleakalae* subgroup	*atrifacies, brunneicrus,* ***ochropleura****,* ***fungiperda****,* ***nigella****, clara, cryptica,* ***haleakalae****, macrochaetae*	We recognize several clades within this subgroup: (1) *fungiperda* complex: ***fungiperda, nigella, nigra, fulgida*** (2) *haleakalae* complex: *clara, cryptica,* ***haleakalae****, macrochaetae* (3) *brunneicrus* complex: *brunneicrus,* ***ochropleura*** (4) *atrifacies* complex: *atrifacies* (5) *venusta* cluster: *inciliata,* ***longiperda****, tanytarsis, venusta* (6) *cilifemorata* cluster: *cilifemorata,* ***dolichotarsis****, flaviceps, stenoptera, swezeyi, chicae, curtitarsis* (7) *iki* complex: ***iki, multiciliata, paraanthrax*** (8) *denotata* complex: *denotata, sabroskyi*
IV.	*luteola* subgroup	*fuscoapex, tamashiroi, luteola, quinqueramosa*	*fuscoapex, tamashiroi, luteola, quinqueramosa*
V.	*polita* subgroup	***bipolita****,* ***canipolita****, dives, flavisternum, illusiopolita, lissodora, mecocnemia,* ***paraanthrax****,* ***polita****, pretiosa*	***bipolita, canipolita****, dives, flavisternum, illusiopolita,* ***insignita****, lissodora, mecocnemia,* ***polita****, pretiosa, chicae, curtitarsis*
VI.	*scitula* subgroup	***fulgida, melanosoma, scitula****, setositibia, subopaca*	This subgroup is not monophyletic. The species sampled have various affinities, none of which are strongly supported. With the exception of *fulgida*, the placement of these species should be considered tentative pending additional work.
VII.	placement uncertain	n/a	***melanosoma, scitula****, setositibia, subopaca*

Species in **bold** were sampled in the current study.

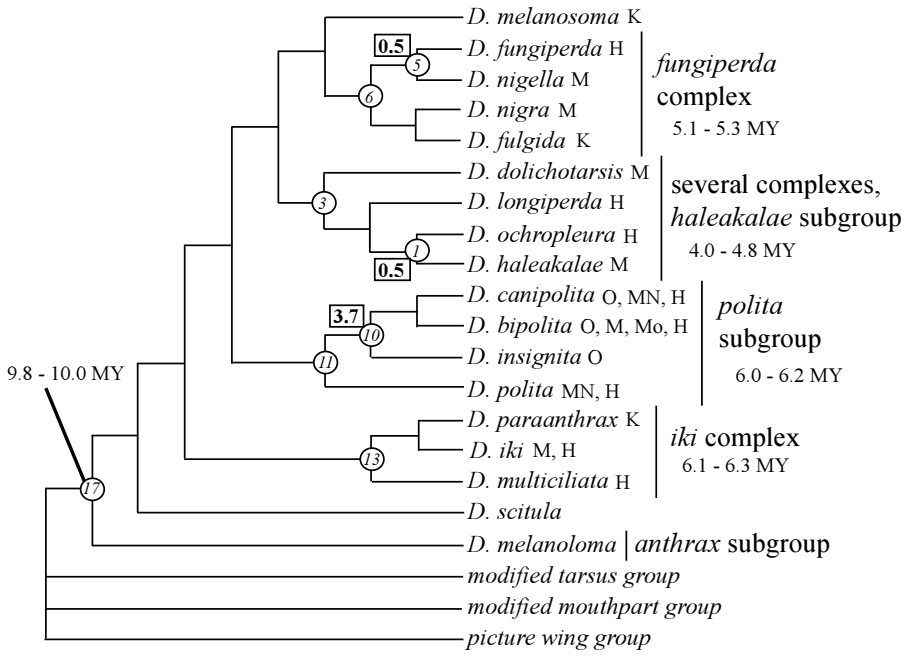

Figure 6. Chronogram showing results of local molecular clock analyses (Yoder & Yang, 2000) based on ML tree topology. Calibration points are in squares and estimated divergence times are placed at various nodes. All ages are in millions of years (MY). Distribution of terminal taxa as follows: K = Kaua'i; O = O'ahu; M = Maui, MN = Maui Nui, Mo = Moloka'i; H = Hawai'i. Revised groupings of species, after Table 5, are also shown. Outgroups have been collapsed into a polytomy.

of the oldest existing high island with suitable rainforest habitat, Kaua'i (5.1 MY). The ages of the internodes connecting these lineages (i.e., nodes 8, 14, 15, 16; Fig. 5) were not estimated because they are very short and poorly supported in the various phylogenetic analyses we have performed (Fig. 4, 5). This suggests that a bottleneck (extinctions or low rates of speciation) prior to the formation of the present day Hawaiian Islands may have taken place in the *haleakalae* species group, as has been suggested in other Hawaiian taxa (Price & Clague, 2002). Such a bottleneck may have been followed by a burst of speciation, giving rise to the present day *haleakalae* group taxa. Additional studies on this and other clades of Hawaiian *Drosophila* will be needed to verify if this pattern is general within this lineage.

One pattern that is not observed in the present data set is the linear progression of taxa found on older high islands to those endemic to younger islands (Bonacum *et al*., *in press*; Hormiga *et al*., 2003; Jordan *et al*., 2003). This is partially due to that fact that taxa in the *haleakalae* species group are reliant on restricted, ephemeral host substrates (fungi) and are quite rare and difficult to sample, making full representation difficult. The other factor is that the taxonomy of this group is difficult to resolve due to few morphological changes between closely related taxa. For example, Hardy *et al*. (2001) listed 16 taxa that were present on multiple islands. This is a rare phenomenon in other Hawaiian groups because of a multitude of sexually selected characters that rapidly change from island to island. Clearly, further genetic studies will be required to better understand the evolutionary forces acting on this and other Hawaiian *Drosophila* groups.

On Hybridization and Gene Choice in Insect Molecular Systematics

Several authors have pointed out problems with using molecular characters to infer phylogenetic relationships when the potential for natural hybridization exists (e.g., Maddison, 1997). In such cases, differential transfer of genetic material can yield incongruent gene trees and combined analysis may result in a biased estimate of species phylogeny. It is unclear whether this may be the case in the *haleakalae* species group as studies of natural hybridization within this group are completely lacking (Kaneshiro and colleagues [Carson *et al.,* 1989; Kaneshiro & Val, 1977; Kaneshiro, 1990] have examined this in other Hawaiian *Drosophila* groups). Furthermore, the individual gene trees obtained here, while not in complete agreement, are not significantly different from one another so it is difficult to tease apart difference due to common ancestry, introgression, or stochastic effects of nucleotide substitution. Further studies within the Hawaiian *Drosophila* examining migration and gene flow within and between populations (and closely related species) are sorely needed before we can adequately address this question. What is clear at this time is that hybridization between closely related taxa, either in the past or in the present day, can obfuscate phylogenetic inference with both molecular and morphological characters.

Molecular systematists working on insects have relied on a standard set of genes, most of which were developed based on previous genetic work in *Drosophila melanogaster*. Gene choice in the past has been driven, at least in part, by what working primers were available, rather than what genes were evolving at the appropriate rate to be potentially informative at the phylogenetic level being examined. A previous study employing *Adh*, *Gpdh*, and 16S (Bonacum, 2001), 3 relatively slowly evolving loci have been widely used in insect systematics, was not particularly effective in resolving relationships among the closely related *haleakalae* group species. Based on some preliminary studies (Zilversmit *et al.*, 2002a), we identified several loci that were evolving rapidly enough to be of use for species-level problems.

This study represents the first application of 5 nuclear genes *Marf, Rpt4, sia, glass,* and *l(2)not-1* to species-level phylogenetic problems. We used 2 criteria, (1) the number of parsimony informative characters and (2) the presence of highly variable, rapidly evolving regions to select loci to resolve relationships among closely related species. The two most influential loci in this study were the *Marf* and *l(2)not*. Both added a significant amount of support to the *haleakalae* phylogeny (Table 4). It is interesting to note that, unlike the slowly evolving *Adh* and *Gpdh* genes, both these protein coding loci do not code for enzymes (*Marf* is a GTP binding protein and *l(2)not* is an integral membrane protein found in membranes of the endoplasmic reticulum). Thus, the gene products of *Marf* and *l(2)not* may be effected by distinctly different selective pressures and, as a result, evolve much more rapidly than enzymatic loci. Homologs of *Marf* and *l(2)not* are present in a wide diversity of Metazoa, suggesting that they may be useful in inferring phylogenetic relationships outside of the Drosophilidae.

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