



Phylogenetic and ecological relationships of the Hawaiian *Drosophila* inferred by mitochondrial DNA analysis

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ABSTRACT

The Hawaiian Drosophilidae are comprised of an estimated 1000 species, all arising from a single common ancestor in the last 25 million years. This group, because of its species diversity, marked sexual dimorphism and complex mating behavior, host plant specificity, and the well-known chronology of the Hawaiian Archipelago, is an excellent model system for evolutionary studies. Here we present a phylogeny of this group based on ~2.6 kb of mitochondrial DNA sequence. Our taxon sampling is the most extensive to date, with nearly 200 species representing all species groups and most subgroups from the larger clades. Our results suggest that the picture wing and modified mouthpart species, long believed to be derived within this radiation, may actually occupy a basal position in the phylogeny. The *haleakale* species group, in contrast, is strongly supported as sister to the AMC clade. We use the phylogenetic results to examine the evolution of two important ecological characters, the host family and type of substrate used for oviposition and larval development. Although both host and substrate transitions are common in the group, oviposition substrate is more conserved among species groups than host plant family. While the ancestral host plant family is equivocally reconstructed, our results suggest that the ancestor of this group may have used rotting bark as a primary oviposition substrate.

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1. Introduction

The Hawaiian Islands have formed over a more-or-less stationary hot spot in the Pacific plate (Price & Clague, 2002; Sharp & Clague, 2006) located in the extreme southeast of the chain. Islands form, migrate slowly to the northwest with the movement of the Pacific plate, and erode, eventually sinking beneath the waves. The Hawaiian hot spot has been active for at least 60 million years and has resulted in a linear progression of islands, with the oldest in the northwest and youngest in the southeast. Currently, the islands can be divided into two main classes: the younger “high islands” with elevations great enough (~1000 m) to capture trade wind moisture and support a diversity of habitats, from subalpine to rainforest to coastal scrub, and those older islands that have subsided and eroded to barely evident atolls, reefs and seamounts (Juvik & Juvik, 1998).

There are six high islands that contain sizeable plant and insect diversity (Kauai, Oahu, Molokai, Lanai, Maui, Hawaii). Perhaps the largest lineage endemic to Hawaiian Archipelago are the

Drosophilidae. These flies comprise a radiation of approximately 1000 species that are thought to be the result of a single colonist lineage that arrived in the islands roughly 25 million years ago (reviewed in Markow & O'Grady, 2006; O'Grady et al., 2009). This group is characterized by high degrees of single island endemism, sexual dimorphism and host plant specificity. A number of authors have posited sexual selection as an explanation for the high species diversity in this group (Carson, 1997), while others have cited geographic subdivision (Carson & Templeton, 1984), host plant specialization (Heed, 1968, 1971; Kambysellis et al., 1995), morphological innovation (Kambysellis, 1993) or a combination of these factors (Craddock, 2000).

Lynn Throckmorton (University of Chicago) used detailed studies of internal morphology to examine phylogenetic relationships among the major lineages of Hawaiian *Drosophila*, providing an evolutionary framework for this group (Throckmorton, 1966, 1975). Throckmorton recognized a number of distinct lineages and referred to them based on male secondary sexual characters. The major lineages include the picture wings (*planitibia*, *grimshawi*, and *adiastola* species groups) and the *modified mouthpart*, *modified tarsus*, *ciliated tarsus*, *haleakalae*, *antopocerus*, *ateledrosophila*, and *nudidrosophila* species groups (Hardy & Kaneshiro, 1969, 1976).

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Several molecular studies have been conducted on the Hawaiian *Drosophila*, either focusing on the placement of the Hawaiian *Drosophila* within the family Drosophilidae (e.g., DeSalle, 1992; Russo, Takezaki, & Nei, 1995), the phylogeny of species groups within the Hawaiian *Drosophila* (Thomas & Hunt, 1991), or relationships among species within a species group (Bonacum, O'Grady, Kambysellis, & Desalle, 2005; O'Grady & Zilversmit, 2004). The majority of these have been in agreement with Thorckmorton's (1975) morphological hypotheses, although some proposed relationships were not strongly supported.

The present study uses mitochondrial DNA to examine phylogenetic relationships within 167 species of Hawaiian *Drosophila*. All known species groups are sampled, making this the largest analysis to date in terms of taxon sampling, and a rigorous test of previous phylogenetic hypotheses. The current data set and analyses allow us to robustly address the following major questions that have lingered in the systematics of the Hawaiian *Drosophila*: the monophyly of all of the major lineages; the placement of these lineages relative to one another; and the phylogenetic relationships within the larger lineages, particularly the *picture wing*, *modified mouthpart*, and *modified tarsus* species. Several significant results of this work are evident, including the (1) placement of the *modified mouthpart*, *picture wing* and related species as basal within the Hawaiian *Drosophila* with the mycophagous *haleakalae* group being sister to the AMC clade, (2) inclusion of the *ciliated tarsus* species group within the *modified tarsus* species group, and (3) paraphyly of the *picture wing* species is a result of the *nudidrosophila* and *ateledrosophila* species groups being nested within the former. We also use our phylogeny to examine the evolution of oviposition preference within the Hawaiian *Drosophila* by focusing on two characters: substrate type (bark and stems, leaves, fruits, etc.) and host plant family.

2. Materials and methods

2.1. Taxon sampling

Of the 167 taxa used in this study, 100 were extracted by the authors for this study. All collections were made via sweeping leaf litter or aspirated directly from sponges baited with fermented banana or rotting mushroom. Specimens were stored in 95% EtOH for identification and DNA extraction. Voucher material was preserved in 95% EtOH. Table 2 includes GenBank Accession numbers, island of origin and the six-digit O'Grady Lab barcode that can be referenced to request more detailed collection information. The subgenus *Scaptomyza* has been identified as sister to the Hawaiian *Drosophila* (O'Grady and DeSalle, 2008), and 18 Hawaiian and mainland species were used as outgroups.

2.2. Sequencing and alignment

Genomic DNA was extracted from single homogenized flies using the Qiagen DNeasy Extraction kit (Qiagen, Inc.). Four mitochondrial genes (Table 1) were amplified. PCR was performed using the following protocol: 5 min initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 5 min. PCR products were cleaned using ExoSAP-IT (USB) following the manufacturer's protocol. Cleaned product was sent to the UC Berkeley DNA Sequencing Facility and sequenced in both directions.

Sequences of additional Hawaiian *Drosophila* species were downloaded from GenBank (Table 2). Sequences for *Drosophila grimshawi* were obtained from the complete genome on Flybase (Tweedie et al., 2009). When multiple sequences for a single

Table 1
Mitochondrial loci used in the current study.

Gene	Length	Primers
16S	513	LR-J-12887: 5'-CCGTTTGAACATCAGATCACGT-3' LR-J-13417: 5'-CGCCTGTTAAACAAAAACAT-3'
Cytochrome oxidase I (COI)	832	2183: 5'-CAACATTTATTTTGATTTTTGG-3' 3037: 5'-TYCATTGCACTAATCTGCCATATTAG-3'
Cytochrome oxidase II (COII)	765	3041: 5'-ATGGCAGATTAGTCAATGG-3' 3791: 5'-GTTTAAGAGACCAGTACTTG-3'
NADH dehydrogenase subunit 2 (ND2)	523	192: 5'-AGCTATTGGGTTACAGACCC-3' 732: 5'-GAAGTTTGGTTAAACCTCC-3'

species were available, only the individual with the most complete sequence coverage was used.

Raw sequences were assembled and edited in Sequencher 4.7 (Gene Codes, Corp.). Assembled sequences were imported into MacClade 4.06 (Maddison & Maddison, 2002) and were aligned by eye using conceptual protein translations. Alignment was trivial for all sequences. A 15 bp non-coding insertion was found between the tRNA-L and COII for a few taxa, and was coded as gaps.

The matrix analyzed was over 80% complete. Table 2 shows sequence coverage per species. COII was sampled from all 167 individuals examined here. Coverage of 16S, COI and ND2 was more variable, ranging from only one to all three genes for each species (Table 2). Genes not sequenced for given individuals were coded as missing. While these analyses contain some missing character information, recent studies have shown that the benefit of increasing taxa outweighs the costs of losing characters in phylogenetic estimation (e.g., Philippe et al., 2004).

2.3. Phylogenetic analyses

A total of 166 taxa, including 18 *Scaptomyza* species, were analyzed to estimate relationships within the Hawaiian *Drosophila*. We employed several analytical methods, including maximum parsimony, maximum likelihood and Bayesian analyses to fully explore this data matrix (Tables 3 and 4). All loci were examined individually and in several combinations. In general, individual analyses were less resolved and lacked statistical support (not shown). Several individual analyses would not finish because the number of taxa exceeded the number of parsimony informative characters (Table 1).

Models of evolution were estimated using MrModeltest 2.3 (Nylander, 2004). The best-fit model of substitution was selected for each locus using the Akaike Information Criterion. The best model for all individual and combined partitions was GTR + I + G. RAXML v7.2.6 (Stamatakis, 2006) was used to estimate the relationships in a maximum likelihood framework. We conducted a maximum likelihood analysis using RAXML-HPC2 on the Abe Teragrid, accessed through the CIPRES portal (Miller et al., 2009). The data was partitioned by gene and the GTR + I + G model was used for each partition (Table 4). Two thousand bootstrap replicates were performed to assess support for the inferred relationships.

A partitioned analysis was run in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) on the Abe Teragrid. Two runs with four chains were run for 10,000,000 generations, sampling trees every 1000 generations (Table 4). The parameters were unlinked and rates were allowed to vary across partitions. Temperature of the chains was set by adjusting the heating parameter in MrBayes to $t = 0.10$ to allow for adequate chain swapping. Convergence was assessed by comparing the average standard deviations of split frequencies, making sure the potential scale reduction factor was effectively 1, and by comparing the cumulative posterior probability of clades in the online program AWTY (Wilgenbusch, Warren, & Swofford, 2004).

Table 2
Taxon sampling.

Species	ID ^a	Island	16S	ND2	COI	COII	Refs. ^b
<i>D. adunca</i>	105818	Maui	EU494374	EU493520	EU493644	EU493902	1
<i>D. aethostoma</i>	109440	Kauai		EU493521	EU493645	EU493774	1
<i>D. ambochila</i>	109433	Oahu		EU493522		EU493776	1
<i>D. ancyla</i>	201019	Maui	HQ170952	HQ170861	HQ170749	HQ170632	8
<i>D. anomalipes</i>	201039	Kauai		HQ170862	HQ170750	HQ170633	8
<i>D. antecedens</i>	205061	Kauai	HQ170953	HQ170863	HQ170751	HQ170634	8
<i>D. apicipuncta</i>	200994	Hawaii	HQ170954		HQ170752	HQ170635	8
<i>D. apodasta</i>	201029	Kauai	HQ170955	HQ170948	HQ170753	HQ170636	8
<i>D. aquila</i>	201000	Hawaii	HQ170956	HQ170864	HQ170754	HQ170637	8
<i>D. atroscutellata</i>	200865	Kauai	HQ171020	HQ170927	HQ170817	HQ170713	8
<i>D. barbata</i>	200996	Hawaii	HQ170957	HQ170865		HQ170638	8
<i>D. basimacula</i>	201030	Kauai	HQ170958	HQ170866	HQ170755	HQ170639	8
<i>D. basisetosa</i>	201021	Hawaii	HQ170959	HQ170867	HQ170756	HQ170640	8
<i>D. bipolita</i>	O75.5	Maui				AY343532	2
<i>D. biseriata</i>	200201	Oahu		HQ170868	HQ170757	HQ170641	8
<i>D. bostrycha</i>	109445	Molokai	EU494378	EU493525	EU493649	EU493778	1
<i>D. brevitarisus</i>	201038	Oahu		HQ170869	HQ170758	HQ170642	8
<i>D. canipolita</i>	O86.2	Hawaii				AY343531	2
<i>D. capitata</i>	201050	Kauai		HQ170870		HQ170643	8
<i>D. chaetocephala</i>	201051	Oahu		HQ170871		HQ170644	8
<i>D. clavitibia</i>	201326	Molokai	HQ170960	HQ170872	HQ170759	HQ170645	8
<i>D. cneucopleura</i>	201022a	Hawaii	HQ170961	HQ170873	HQ170760	HQ170646	8
<i>D. comatifemora</i>	109459	Maui	EU494380	EU493527	EU493651	EU493780	1
<i>D. conformis</i>	201312a	Hawaii	HQ171023	HQ170930	HQ170820	HQ170716	8
<i>D. contorta</i>	200120a	Maui	HQ171021	HQ170928	HQ170818	HQ170714	8
<i>D. cracens</i>	O56.8	Hawaii	HQ171037		HQ170834	HQ170731	3, 8
<i>D. crucigera</i>	hd, W41N3	Oahu				AY343526	2
<i>D. cyrtoloma</i>	201798a	Maui	HQ170962	HQ170874	HQ170761	HQ170647	8
<i>D. dasycnemia</i>	202326b	Hawaii	HQ171026	HQ170933	HQ170823	HQ170719	8
<i>D. desallei</i>	200989	Hawaii	HQ170963	HQ170875	HQ170762	HQ170648	8
<i>D. diamphidiopoda</i>	O35.2	Maui	HQ171029		HQ170826	HQ170724	3, 8
<i>D. differens</i>	KYK	Molokai	AY006397		HQ170840	AY006435	3, 5, 8
<i>D. disjuncta</i>		Maui	U94256			U94213	4
<i>D. dissita</i>	201323	Hawaii	HQ170964	HQ170876	HQ170763	HQ170649	8
<i>D. dolomata</i>	200999	Oahu	HQ170965		HQ170764	HQ170650	8
<i>D. eurypeza</i>	201043	Kauai	HQ170966	HQ170944	HQ170765	HQ170651	8
<i>D. expansa</i>	201012	Maui	HQ170967	HQ170877	HQ170766	HQ170652	8
<i>D. fastigata</i>	200866	Oahu	HQ171019	HQ170926	HQ170816	HQ170712	8
<i>D. flavibasis</i>	205062	Kauai	HQ170968	HQ170878	HQ170767	HQ170653	8
<i>D. forficata</i>	201053	Kauai		HQ170879		HQ170654	8
<i>D. fulgida</i>	O87.9	Kauai				AY343529	2
<i>D. fundita</i>	201023	Maui	HQ170969	HQ170880	HQ170768	HQ170655	8
<i>D. fungiperda</i>	O38.5	Hawaii				AY343535	2
<i>D. furcatarsus</i>	201054	Kauai		HQ170881		HQ170656	8
<i>D. furvifacies</i>	200209	Oahu	HQ170970	HQ170882		HQ170657	8
<i>D. fuscoamoeba</i>	200977	Oahu				HQ170658	8
<i>D. glabriapex</i>	109446	Kauai	EU494384	EU493531	EU493655	EU493784	1
<i>D. grimshawi</i>	hd, G40	Maui	BK006341	BK006341	BK006341	BK006341	6, 7
<i>D. haleakalae</i>	109330	Maui		EU493532		EU493785	1
<i>D. hamifera</i>	109435	Maui	EU494267		EU493657	EU493786	1
<i>D. hanaulae</i>	MPK	Maui	AY006389		HQ170841	AY006427	3, 5, 8
<i>D. hemipeza</i>	RD	Oahu	AY006391		HQ170842	AY006429	3, 5, 8
<i>D. heteroneura</i>	hd, W33B4	Hawaii	AY006434		HQ170843	AY006434	3, 5, 8
<i>D. hirtitibia</i>	109429	Oahu	EU494387	EU493533	EU493658	EU493787	1
<i>D. hystriosa</i>	109444	Maui	EU494388	EU493534	EU493659	EU493788	1
<i>D. iki</i>	MPK	Hawaii	HQ171032		HQ170829	HQ170727	3, 8
<i>D. imparisetae</i>	201014	Hawaii	HQ170971	HQ170883	HQ170769	HQ170659	8
<i>D. inciliata</i>	hd, Y33	Maui	HQ171033		HQ170830	HQ170728	3, 8
<i>D. incognita</i>	202333a	Hawaii	HQ171027	HQ170934	HQ170824	HQ170720	8
<i>D. infuscata</i>	205063	Hawaii	HQ170972	HQ170884	HQ170770	HQ170660	8
<i>D. ingens</i>	hd, Y1862	Maui	AY006406			AY006444	5
<i>D. insignita</i>	O86.1	Oahu				HQ170723	2, 8
<i>D. involuta</i>	205064	Hawaii	HQ170973	HQ170885	HQ170771	HQ170661	8
<i>D. ischnotrix</i>	200990a	Oahu	HQ170974	HQ170886		HQ170662	8
<i>D. kambysellisi</i>	205065a	Hawaii	HQ170975	HQ170949	HQ170772	HQ170663	8
<i>D. kauluai</i>	205072	Oahu		HQ170887	HQ170773	HQ170664	8
<i>D. kokeensis</i>	201055	Kauai		HQ170888		HQ170665	8
<i>D. konaensis</i>	O39.2	Hawaii	HQ171038		HQ170835	HQ170732	3, 8
<i>D. kualapa</i>	201046	Kauai	HQ170976	HQ170889	HQ170774	HQ170666	8
<i>D. latigena</i>	201015	Hawaii	HQ170977	HQ170890	HQ170775	HQ170667	8
<i>D. lineosetae</i>	hd, Y5	Maui	HQ171039		HQ170836	HQ170733	3, 8
<i>D. longiperda</i>	hd, Y5	Hawaii	HQ171034		HQ170831	HQ170729	3, 8
<i>D. longiseta</i>	O58.D	Molokai	HQ171030		HQ170827	HQ170725	3, 8
<i>D. malele</i>	201047	Kauai	HQ170978	HQ170891	HQ170776	HQ170668	8

Table 2 (continued)

Species	ID ^a	Island	16S	ND2	COI	COII	Refs. ^b
<i>D. medialis</i>	201017	Hawaii	HQ170979	HQ170892	HQ170777	HQ170669	8
<i>D. melanocephala</i>	201799	Maui	HQ170980		HQ170778	HQ170670	8
<i>D. melanoloma</i>	105708	Maui	EU494391	EU493536	EU493662	EU493791	1
<i>D. micromyia</i>	201044	Kauai	HQ170981	HQ170893	HQ170779	HQ170671	8
<i>D. mimica</i>	205066a	Hawaii	HQ170982	HQ170950	HQ170780	HQ170672	8
<i>D. mitchelli</i>	202312	Hawaii	HQ170983	HQ170894	HQ170781	HQ170673	8
<i>D. multiciliata</i>	109439	Hawaii	EU494393	EU493538		EU493794	1
<i>D. nanella</i>	201042	Kauai	HQ170984	HQ170895	HQ170782	HQ170674	8
<i>D. neoperkinsi</i>	MPK	Molokai	AY006398		HQ170844	AY006436	3, 5, 8
<i>D. neopicta</i>	O41.C	Maui	AY006401			AY006439	5, 8
<i>D. neutralis</i>	202319b	Hawaii	HQ171024	HQ170931	HQ170821	HQ170717	8
<i>D. nigella</i>	105820	Maui		EU493539	EU493666	EU493795	1
<i>D. nigra</i>	105821	Maui	EU494394	EU493540	EU493667	EU493796	1
<i>D. nigribasis</i>	MPK	Oahu	AY006394		HQ170845	AY006432	3, 5, 8
<i>D. nigrocirrus</i>	200993	Hawaii	HQ170985	HQ170896	HQ170783	HQ170675	8
<i>D. oahuensis</i>	RD	Oahu	AY006393		HQ170846	AY006431	3, 5, 8
<i>D. obscuripes</i>	hd, Y50	Maui	AY006405			AY006443	5, 8
<i>D. ochracea</i>	109447	Hawaii	EU494395	EU493541		EU493797	1
<i>D. ochropleura</i>	O84.1	Hawaii				AY343530	2
<i>D. papaalai</i>	201045	Kauai	HQ170986	HQ170897	HQ170784	HQ170676	8
<i>D. paraanthrax</i>	O89.2	Kauai				HQ170722	2, 8
<i>D. paucitarsus</i>	201024	Molokai	HQ170987	HQ170898	HQ170785	HQ170677	8
<i>D. pectinitarsus</i>	201025	Hawaii	HQ170988	HQ170899	HQ170786	HQ170678	8
<i>D. percnosoma</i>	200125a	Hawaii	HQ171022	HQ170929	HQ170819	HQ170715	8
<i>D. perissopoda</i>	201034	Kauai	HQ170989	HQ170945	HQ170787	HQ170679	8
<i>D. petalopeza</i>	O41.2	Maui	HQ171036		HQ170833	HQ170730	3, 8
<i>D. picticornis</i>	MPK	Kauai	AY006392		HQ170847	AY006430	3, 5, 8
<i>D. planitibia</i>	hd, U84 N	Maui	AY006400		HQ170848	AY006438	3, 5, 8
<i>D. polita</i>	hd, Y56	Lanai	HQ171035		HQ170832	AY343538	2, 3, 8
<i>D. polliciforma</i>	200976	Hawaii	HQ170990	HQ170900	HQ170788	HQ170680	8
<i>D. poonia</i>	201048	Kauai	HQ170991		HQ170789	HQ170681	8
<i>D. preapicula</i>	201040	Hawaii	HQ170992	HQ170901	HQ170790	HQ170682	8
<i>D. primaeva</i>	200863	Kauai	HQ170993		HQ170791	HQ170683	8
<i>D. procerisetae</i>	201880	Molokai	HQ170994	HQ170902	HQ170792	HQ170684	8
<i>D. prodita</i>	201010	Hawaii	HQ170995	HQ170903	HQ170793	HQ170685	8
<i>D. prolactilia</i>	O85.6	Hawaii	HQ171040		HQ170837	HQ170734	3, 8
<i>D. propiofacies</i>	201027	Maui	HQ170996	HQ170904	HQ170794	HQ170686	8
<i>D. pullipes</i>	109448	Hawaii			EU493671	EU493800	1
<i>D. pychnochaetae</i>	200991	Oahu	HQ170997	HQ170905	HQ170795	HQ170687	8
<i>D. redunda</i>	201884	Molokai	HQ170998	HQ170906	HQ170796	HQ170688	8
<i>D. reschae</i>	205073	Oahu	HQ170797		HQ170689	HQ170999	8
<i>D. scitula</i>	O87.E	Kauai				AY343533	2
<i>D. seclusa</i>	201011	Hawaii	HQ171000	HQ170907	HQ170798	HQ170690	8
<i>D. setosifrons</i>	hd, Y80	Hawaii	AY006404		HQ170849	AY006442	3, 5, 8
<i>D. silvestris</i>	hd, W34B4	Hawaii	AY006395		HQ170850	AY006433	3, 5, 8
<i>D. sodomae</i>	O34.2	Molokai	HQ171041		HQ170838	HQ170746	3, 8
<i>D. soonae</i>	109458	Hawaii	EU494398	EU493544	EU493672	EU493801	1
<i>D. sordidapex</i>	202321a	Hawaii	HQ171025	HQ170932	HQ170822	HQ170718	8
<i>D. substenoptera</i>	MPK	Oahu	AY006402		HQ170851	AY006440	3, 5, 8
<i>D. systemopeza</i>	201026	Maui	HQ171014	HQ170920	HQ170812	HQ170706	8
<i>D. tanythrix</i>	hd, Y48	Hawaii	HQ171031		HQ170828	HQ170726	3, 8
<i>D. tetraspilota</i>	200995	Hawaii	HQ171015	HQ170921		HQ170707	8
<i>D. toxacantha</i>	200864	Hawaii		HQ170922		HQ170708	8
<i>D. trichaetosa</i>	201013	Hawaii	HQ171016	HQ170923	HQ170813	HQ170709	8
<i>D. unacula</i>	200997	Hawaii	HQ171017	HQ170924	HQ170814	HQ170710	8
<i>D. villosepedis</i>	O61.2	Kauai	HQ171042		HQ170839	HQ170747	3, 8
<i>D. waddingtoni</i>	000096	Hawaii	HQ171028	HQ170935	HQ170825	HQ170721	8
<i>D. xenophaga</i>	205070	Hawaii	HQ171018	HQ170925	HQ170815	HQ170711	8
" <i>D. spKNMa</i> "	201325a	Hawaii				HQ170691	8
" <i>D. nr ancyla</i> "	201020	Hawaii	HQ171007	HQ170913	HQ170805	HQ170698	8
" <i>D. nr basimacula 1</i> "	201031	Kauai	HQ171001	HQ170951	HQ170799	HQ170692	8
" <i>D. nr basimacula 2</i> "	201032	Kauai	HQ171003	HQ170909	HQ170801	HQ170694	8
" <i>D. nr basimacula 3</i> "	201033	Kauai	HQ171005	HQ170911	HQ170803	HQ170696	8
" <i>D. nr brunneisetae</i> "	201018	Maui	HQ171008	HQ170914	HQ170806	HQ170699	8
" <i>D. nr dorsigera</i> "	201052	Kauai		HQ170946		HQ170700	8
" <i>D. nr fundita</i> "	201028	Hawaii	HQ171009	HQ170915	HQ170807	HQ170701	8
" <i>D. nr hirtitarsus</i> "	200998	Kauai	HQ171010	HQ170916	HQ170808	HQ170702	8
" <i>D. nr humeralis</i> "	201041	Kauai	HQ171011	HQ170917	HQ170809	HQ170703	8
" <i>D. nr melanopedis</i> "	201016	Hawaii	HQ171012	HQ170918	HQ170810	HQ170704	8
" <i>D. nr perissopoda 1</i> "	201035	Kauai	HQ171002	HQ170908	HQ170800	HQ170693	8
" <i>D. nr perissopoda 2</i> "	201036	Kauai	HQ171004	HQ170910	HQ170802	HQ170695	8
" <i>D. nr perissopoda 3</i> "	201037	Kauai	HQ171006	HQ170912	HQ170804	HQ170697	8
" <i>D. nr residua</i> "	201324	Hawaii	HQ171013	HQ170919	HQ170811	HQ170705	8
" <i>S. akalae</i> "	201528	Hawaii	HQ171045		HQ170854	HQ170736	8
<i>S. albovittata</i>		Oahu	U94262			U94220	4

(continued on next page)

Table 2 (continued)

Species	ID ^a	Island	16S	ND2	COI	COII	Refs. ^b
<i>S. anomala</i>	hd, G32C1	Oahu	HQ171043		HQ170852	HQ170748	3, 8
<i>S. caliginosa</i>	105680	Hawaii	EU494402		EU493676	EU493805	1
<i>S. crassifemur</i>	109449	Hawaii		EU493547	EU493677	U94211	1
<i>S. cyrtandrae</i>	201201	Hawaii	HQ171046	HQ170936		HQ170737	8
<i>S. elmoi</i>	hd, K22A107	Oahu	HQ171044		HQ170853	HQ170735	3, 8
<i>S. flava</i>	201322	Washington	HQ171047	HQ170937	HQ170855	HQ170738	8
<i>S. frustulifera</i>	108518	Gough Is.	EU494405	EU493549	EU493679	EU493808	1
<i>S. longisetosa</i>	201057a	Hawaii		HQ170938	HQ170856	HQ170739	8
<i>S. magnipalpa</i>	201056	Kauai	HQ171048	HQ170947	HQ170857	HQ170740	8
" <i>S. neocyrtandrae</i> "	201368	Mauai	HQ171049	HQ170939		HQ170741	8
<i>S. palmae</i>	106323	Kauai	EU494406	EU493550	EU493680	EU493809	1
<i>S. remota</i>	108519	Gough Is.			EU493675	EU493804	1
<i>S. silvicola</i>	201061	Hawaii		HQ170940	HQ170858	HQ170742	8
<i>S. tumidula</i>	201059a	Hawaii	HQ171050	HQ170941		HQ170743	8
<i>S. undulata</i>	109452	Hawaii	EU494407	EU493551	EU493681	EU493810	1
<i>S. varifrons</i>	201058	Oahu		HQ170942	HQ170859	HQ170744	8
<i>S. varipicta</i>	201060a	Kauai	HQ171051	HQ170943	HQ170860	HQ170745	8

^a Collection information. hd, alphanumeric code: Hawaiian *Drosophila* Project Collection, KYK: Kenneth Y. Kaneshiro Collection, MPK: Michael P. Kambysellis Collection, O#: O'Grady Lab Collection, RD: Rob DeSalle Collection.

^b References. 1. O'Grady and DeSalle (2008), 2. O'Grady and Zilversmit (2004), 3. Bonacum (2001), 4. Baker and DeSalle (1997), 5. Bonacum et al. (2005), 6. Clark et al. (2007); 7. Montooth et al. (2009), 8. This study.

Table 3

Summary of maximum parsimony analyses.

	# Taxa	# Chars (PI)	# Trees (length)
<i>Individual</i>			
16S	134	513 (70)	^a
COI	132	832 (280)	^a
COII	166	765 (282)	^a
ND2	111	523 (187)	1644 (1560)
<i>Combined</i>			
All 4	83	2633 (687)	3 (5245)
3 of 4	138	2633 (798)	2 (8066)
2 of 4	155	2633 (814)	106 (8623)
All data	166	2633 (819)	115 (8823)

^a Search would not finish.

2.4. Character mapping

MacClade (Maddison & Maddison, 2002) was used to perform character mapping. Data on substrate type (fungi, leaves, bark or stem, fruit, sap flux) and host plant (fungi, Araliaceae, Campanulaceae, ferns, Nyctagenaceae, Amaranthaceae, Sapindaceae, Fabaceae, Myrsinaceae, Aquifoliaceae and other) were entered based on Magnacca, Foote, and O'Grady (2008). When multiple records existed, we selected those with multiple rearings or multiple offspring. Incidental rearing records, those with single plant records or one to a few offspring, were not coded. Two taxa, *D. grimshawi* and *Drosophila crucigera*, were coded as generalists because they had been reared multiple times from many different hosts.

Table 4

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

Gene	Model	−ln L	G ^a	I ^b	Base frequencies	Rate matrix			
ND2	GTR + I + G	6265.9751	0.5806	0.4510	A = 0.3717	G = 0.0580	A–C = 1.2304	A–T = 2.0052	C–T = 16.9635
					C = 0.1168	T = 0.4536	A–G = 29.6464	C–G = 3.3124	G–T = 1.0000
COI	GTR + I + G	10078.1377	0.6607	0.5966	A = 0.3675	G = 0.0659	A–C = 0.4969	A–T = 0.9674	C–T = 11.7876
					C = 0.1119	T = 0.4547	A–G = 16.1613	C–G = 0.6713	G–T = 1.0000
COII	GTR + I + G	10154.0195	0.5321	0.5379	A = 0.3750	G = 0.0688	A–C = 1.9311	A–T = 3.4463	C–T = 59.8773
					C = 0.1059	T = 0.4502	A–G = 31.4295	C–G = 3.4072	G–T = 1.0000
16S	GTR + I + G	1898.3174	0.6233	0.6985	A = 0.4057	G = 0.0820	A–C = 0.0000	A–T = 2.8979	C–T = 11.0933
					C = 0.1472	T = 0.3650	A–G = 6.0256	C–G = 0.5550	G–T = 1.0000

^a Gamma shape parameter.

^b Proportion of invariant sites.

3. Results

3.1. Higher-level phylogenetic relationships in Hawaiian *Drosophila*

Fig. 1 summarizes the phylogenetic relationships among the major lineages of Hawaiian *Drosophila*. This phylogeny shows a close association between the *antopocerus*, *modified tarsus*, and *ciliated tarsus* species (PP = 100, BP = 89; Fig. 1B, AMC Clade), as observed in some earlier studies (Bonacum, 2001). Our results suggest that, contrary to previous studies, the *haleakale* species group is not the basal-most branch in the Hawaiian *Drosophila*. This group is sister to the AMC clade with strong support (PP = 100, BP = 89; Fig. 1B). The modified mouthpart species group is monophyletic (PP = 73, Fig. 1B) and sister to the AMC–haleakalae species (PP = 73, Fig. 1A). The *picture wing*, *nudidrosophila*, and *ateledrosophila* species form a clade (PNA clade) with only modest support (PP = 71, Fig. 1A). The PNA clade is the most basal lineage in this phylogeny.

3.2. Relationships within species groups and clades

3.2.1. The PNA clade

The picture wing species group currently contains 114 described species, although this group is paraphyletic and that number increases to 145 when members the *nudidrosophila* and *ateledrosophila* species groups are included. Within the picture wings (Fig. 1A), our analyses supported the monophyly of the

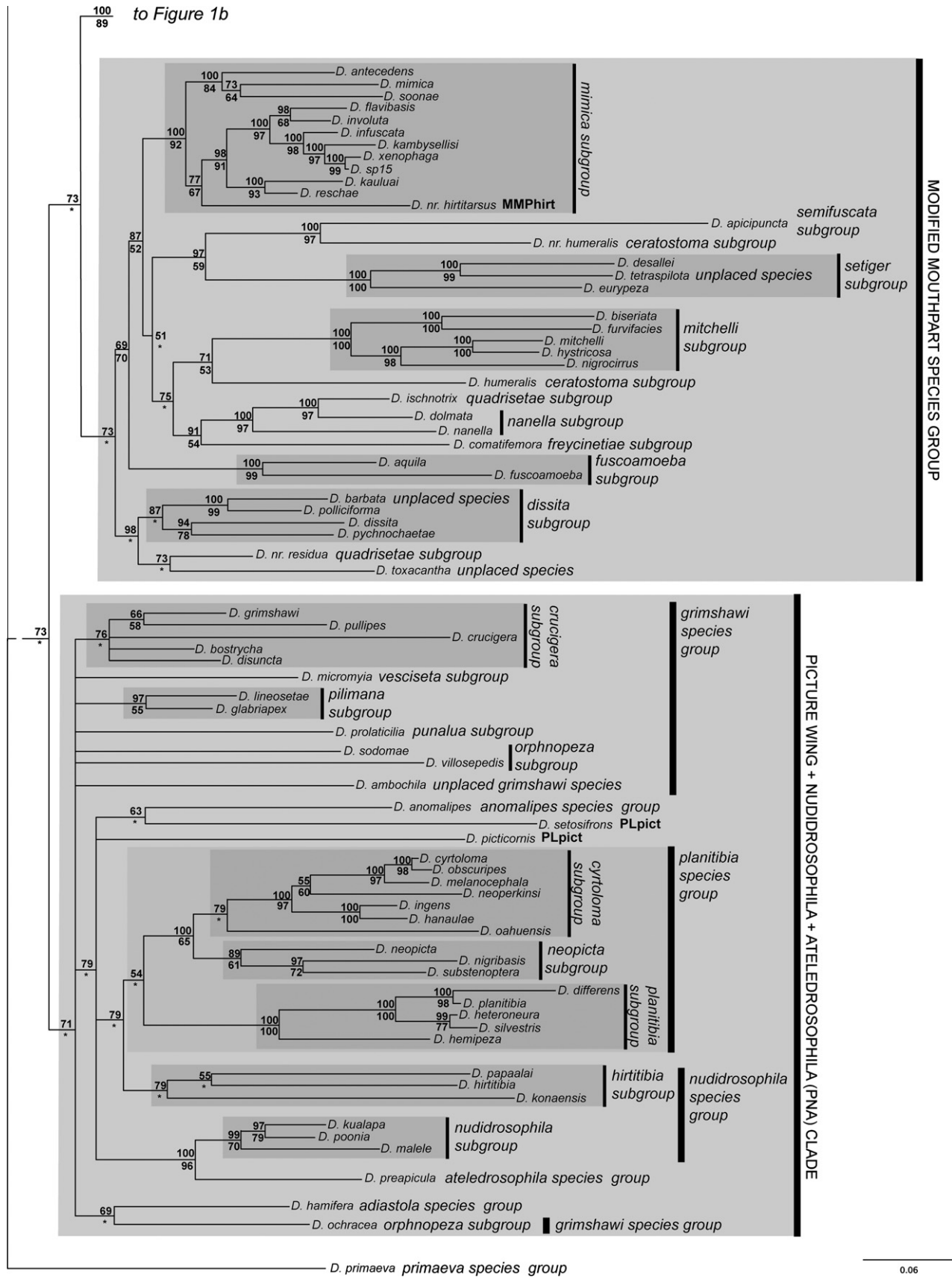
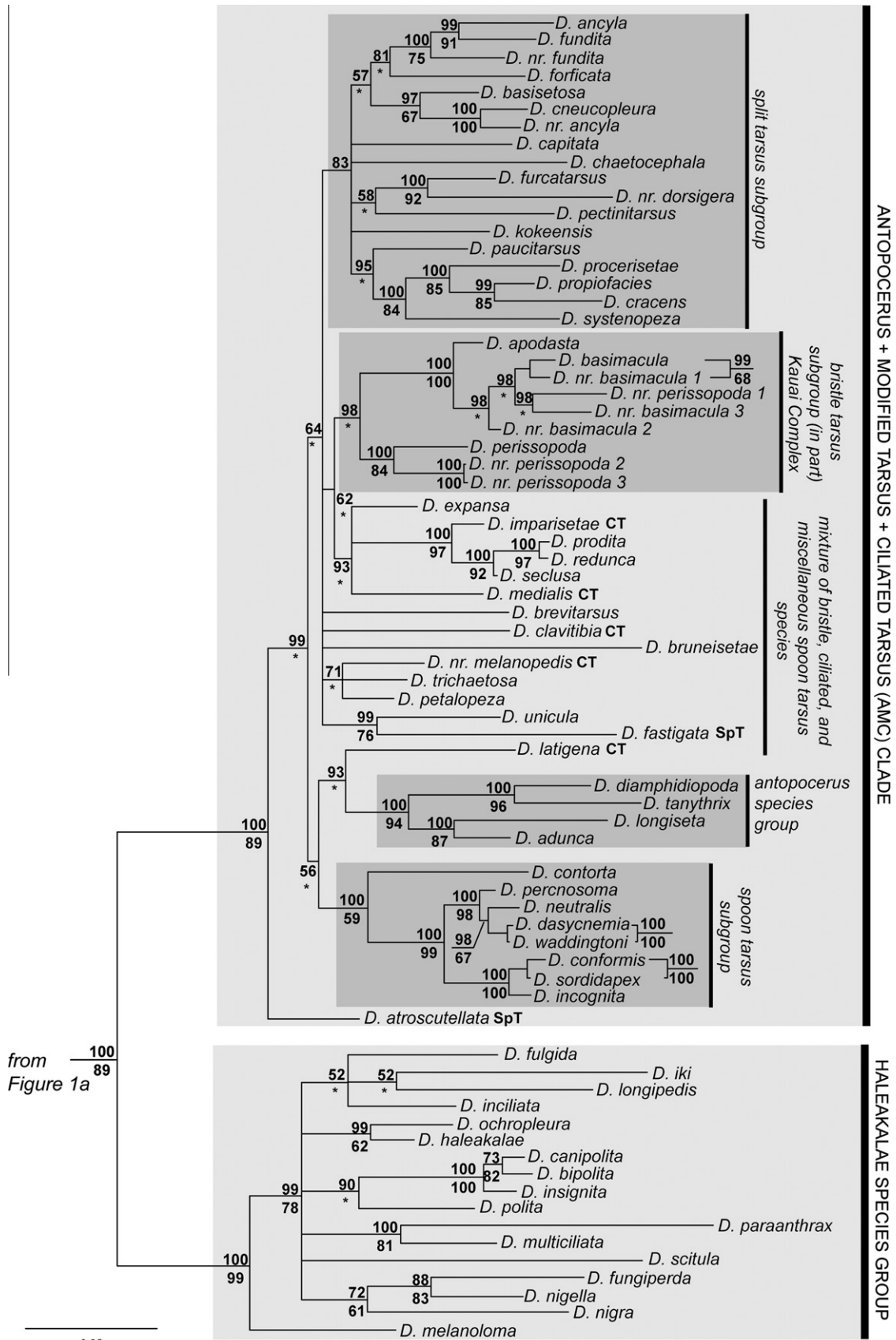


Fig. 1. Bayesian analysis of combined mitochondrial data matrix showing relationships among species, species subgroup and species groups within the Hawaiian *Drosophila*. Posterior probabilities are above each node, maximum likelihood bootstrap values are below, an * indicates relationships with less than 50% support. A. Phylogeny of basal lineages in the picture wing, *nuddrosophila*, *ateledrosophila*, and modified mouthpart groups. B. Phylogeny of *haleakalae*, modified tarsus, and *antopocerus* species groups.



from $\frac{100}{89}$ Figure 1a

Fig. 1 (continued)

cyrtoloma (PP = 79), *neopicta* (PP = 89, BP = 61), *planitibia* (PP = 100, BP = 100), *crucigera* (PP = 76), and *pilimana* (PP = 97) subgroups. There was also weak support for the *planitibia* species group (PP = 54), although this lacked the *picticornis* subgroup. The *nudidrosophila* species group was not monophyletic (Fig. 1A), with species in the *hirtitibia* subgroup (PP = 79) sister to the *planitibia* subgroup with weak support (PP = 79) and those in the *nudidrosophila* subgroup (PP = 99, BP = 70) strongly supported as sister to the *ateledrosophila* species group (PP = 100, BP = 96). No strong support was recovered for most relationships within the *grimshawi* species group (Fig. 1A), a large clade that needs additional sampling before a picture of phylogenetic relationships will emerge.

3.2.2. The modified mouthparts species group

With 106 described species (O'Grady et al., 2010), this is the second largest group of Hawaiian *Drosophila*. However, given the large numbers of undescribed species present in collections at UC Berkeley, UH Manoa and the BP Bishop Museum (Magnacca & O'Grady, 2009), this group may be considerably larger than the PNA clade. A number of recent studies (O'Grady et al., 2003, Magnacca & O'Grady, 2006, 2009) have provided a taxonomic structure within this group that can be tested with phylogenetic analyses. The *mitchelli* (PP = 100, BP = 100) and *fuscoamoeba* (PP = 100, BP = 99) subgroups are both monophyletic (Fig. 1A), although only two taxa are sampled from the *fuscoamoeba* species and additional work will be needed to rigorously test the composition of this group. The *setiger* subgroup (Fig. 1A) forms a clade (PP = 100, BP = 100) with *D. tetraspilota*, a previously unplaced species. The *dissita* group (Fig. 1A) also contains an unplaced species, *D. barbata*, and is only moderately supported (PP = 87) as monophyletic. The *mimica* subgroup is not monophyletic (Fig. 1A) and includes a single member of the *hirtitarsus* subgroup, a new species closely related to *D. hirtitarsus*. It is possible that the definition of the *mimica* subgroup should be expanded to include the two species placed in the *hirtitarsus* subgroup. The *ceratostoma* subgroup (Magnacca & O'Grady, 2006) was not monophyletic in our analyses, with the two sampled species being either sister to the *semifuscata* or *mitchelli* subgroups (Fig. 1A). A well-supported clade of species in the *freycinetiae*, *nanella* and *quadrisetae* subgroups was recovered in this analysis (PP = 91, BP = 54; Fig. 1A). Reconstitution of these groups may be necessary because of the close phylogenetic relationships and the paraphyly of the *nanella* subgroup. Additional sampling from within this complex group of species will be needed before the phylogeny of the modified mouthpart species can be considered finished.

3.2.3. The AMC clade

Our results show strong support for the monophyly of the *antopocerus*, *modified tarsus* and *ciliated tarsus* species groups (PP = 100, BP = 89; Fig. 1B). Within this clade there are several established species groups and subgroups that are monophyletic, such as the *antopocerus* (PP = 100, BP = 94; Fig. 1B) and *split tarsus* (PP = 83, Fig. 1B) species groups. Other lineages are clearly polyphyletic. For example, the *spoon tarsus* subgroup as it is currently defined (*sensu* Lapoint, Magnacca, & O'Grady, 2009) is not monophyletic. Lapoint, Magnacca, & O'Grady (2009) included two taxa, *D. atroscutellata* and *D. fastigata*, within the spoon tarsus subgroup. Our results indicate that these two species not related to one another or close to the spoon tarsus subgroup. *Drosophila atroscutellata* is basal to the entire AMC clade (PP = 99, Fig. 1B) and *D. fastigata* is sister to *D. uncula* (PP = 99; BP = 76) and embedded within a paraphyletic mixture of bristle and ciliated tarsus species. It should be noted, however, that species traditionally included in the spoon tarsus group do form a clade (PP = 100, BP = 59; Fig. 1B) and this group should be reconstituted to reflect the recent phylogenetic work. The *bristle* and *ciliated tarsus* species form a

paraphyletic grade of taxa sister to the *split tarsus* species group. While additional taxonomic and phylogenetic work will be required to address the relationships among these taxa, there is support for a complex of Kauai endemic species in the *bristle tarsus* species group (PP = 98, Fig. 1B).

3.2.4. The haleakalae species group

This species group is strongly supported as monophyletic (PP = 100, BP = 99; Fig. 1B) and sister to the AMC clade (PP = 100, BP = 89; Fig. 1B). Hardy, Kaneshiro, Val, and O'Grady (2002) proposed a number of species subgroups and complexes for this group, few of which are supported as monophyletic in this or previous (O'Grady and Zilversmit, 2004) studies. Two complexes that are supported in the present study are the *fungiperda* complex (PP = 72, BP = 61; Fig. 1B) and some species in the *polita* complex (PP = 90, Fig. 1B). Additional work, focusing specifically on this rarely collected group of mycophagous species will be required before the taxonomic and phylogenetic relationships within this group can be clarified.

3.3. The evolution of host use

Fig. 2 shows the results of character mapping using MacClade (Maddison & Maddison, 2002). We examined two different components of host use, the type of substrate that females oviposit and larvae develop in, and, the host plant family that a given species utilizes. In general, host plant family use appears to be much more labile than substrate type, particularly within the basal PNA clade and *modified mouthpart* species group (Fig. 2). In contrast, substrate type tends to be conserved within clades (Kambysellis et al., 1995). Utilization of bark appears to be the ancestral state within the Hawaiian *Drosophila* as many species in the PNA clade and *modified mouthpart* species group using this resource (Fig. 2A). The transition to fungus or leaf breeding occurred at the base of the *haleakalae* species group and AMC clade, respectively (Fig. 2A). Several parallel transitions in substrate use have occurred, including at least four shifts to leaves and three shifts to sap fluxes. Reversals are also common, with several transitions back to bark breeding within the AMC clade (Fig. 2A).

While Araliaceae and Campanulaceae are the most commonly used host plant families (Magnacca, Foote, & O'Grady, 2008), switches between host plant families occurs more frequently, with many shifts occurring within a given species group or even species subgroup (Fig. 2B). The exception to this seems to be in the AMC clade, where most species utilize Araliaceae, with several independent transitions to Campanulaceae, Nyctaginaceae or other less common host families (Fig. 2B).

4. Discussion

4.1. Taxon sampling and outgroup selection

The major differences between the current study and previous molecular analyses are ingroup sampling and outgroup selection. The more comprehensive earlier studies (e.g., Kambysellis et al., 1995) did not sample any *haleakale* species. Others (i.e., Baker & DeSalle, 1997; Thomas and Hunt, 1991, 1993) only sampled a few representative taxa (Fig. 3C). Bonacum's (2001) sampling was the most extensive of the previous studies and, while the *haleakalae* group was basal (Fig. 3D), support for this position in some analyses was weak. O'Grady and DeSalle (2008) examined broader-scale patterns in the *Drosophilidae*, but included extensive samples within the Hawaiian *Drosophila*. The *haleakalae* group was basal, but with only moderate support.

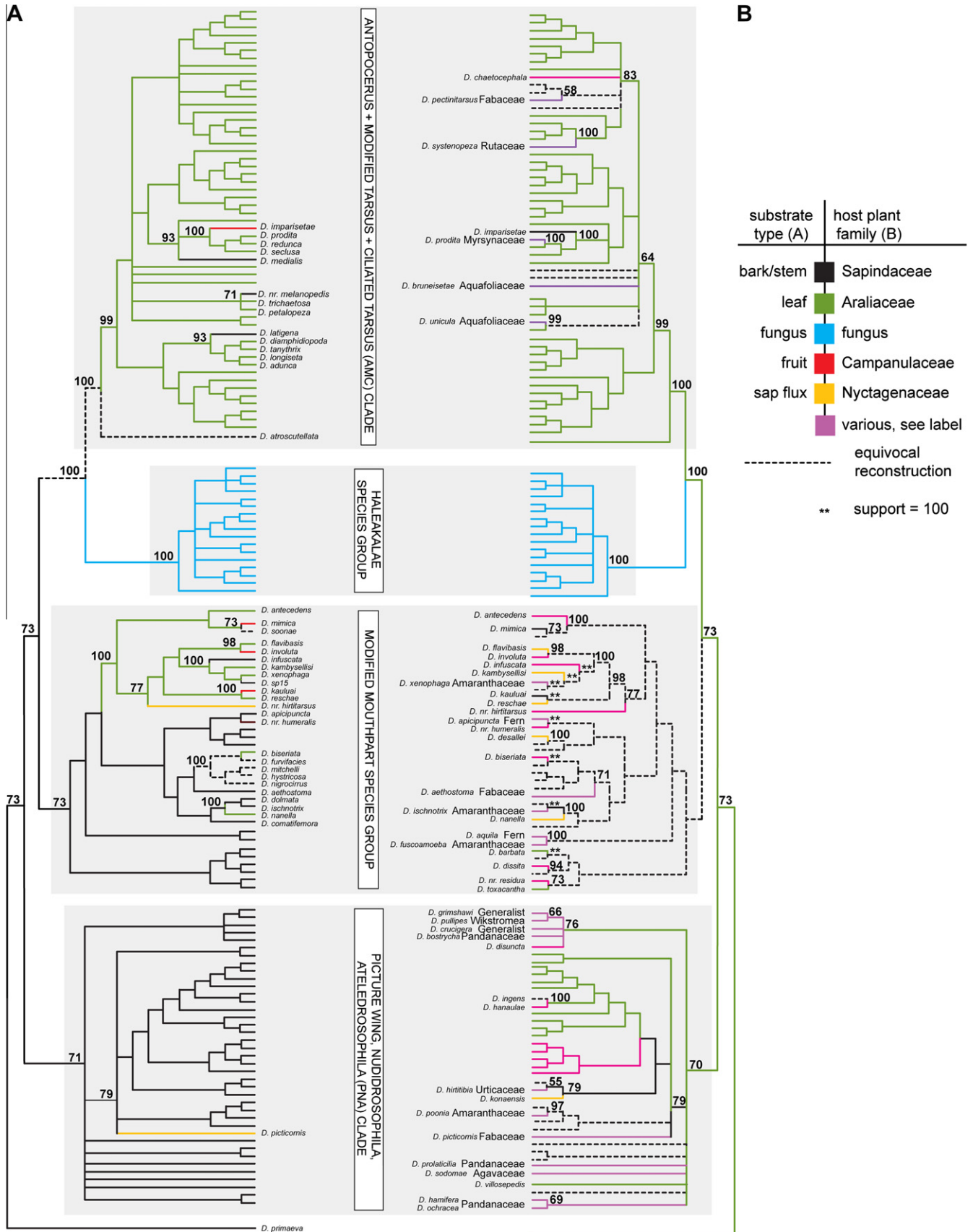


Fig. 2. Character mapping of ecological data (Magnacca, Foote, & O'Grady, 2008). A. Type of substrate that larvae utilize: stems and bark (black), leaves (green), fungus (blue), fruit (red), sap flux (orange). Equivocal reconstructions are indicated with a dashed line. B. Host plant family utilized by larvae: Sapindaceae (black), Araliaceae (green), fungus (blue), Campanulaceae (red), Nyctagenaceae (orange), various (purple, exact family is labeled on figure). Equivocal reconstructions are indicated with a dashed line, ** indicates a support value of 100%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

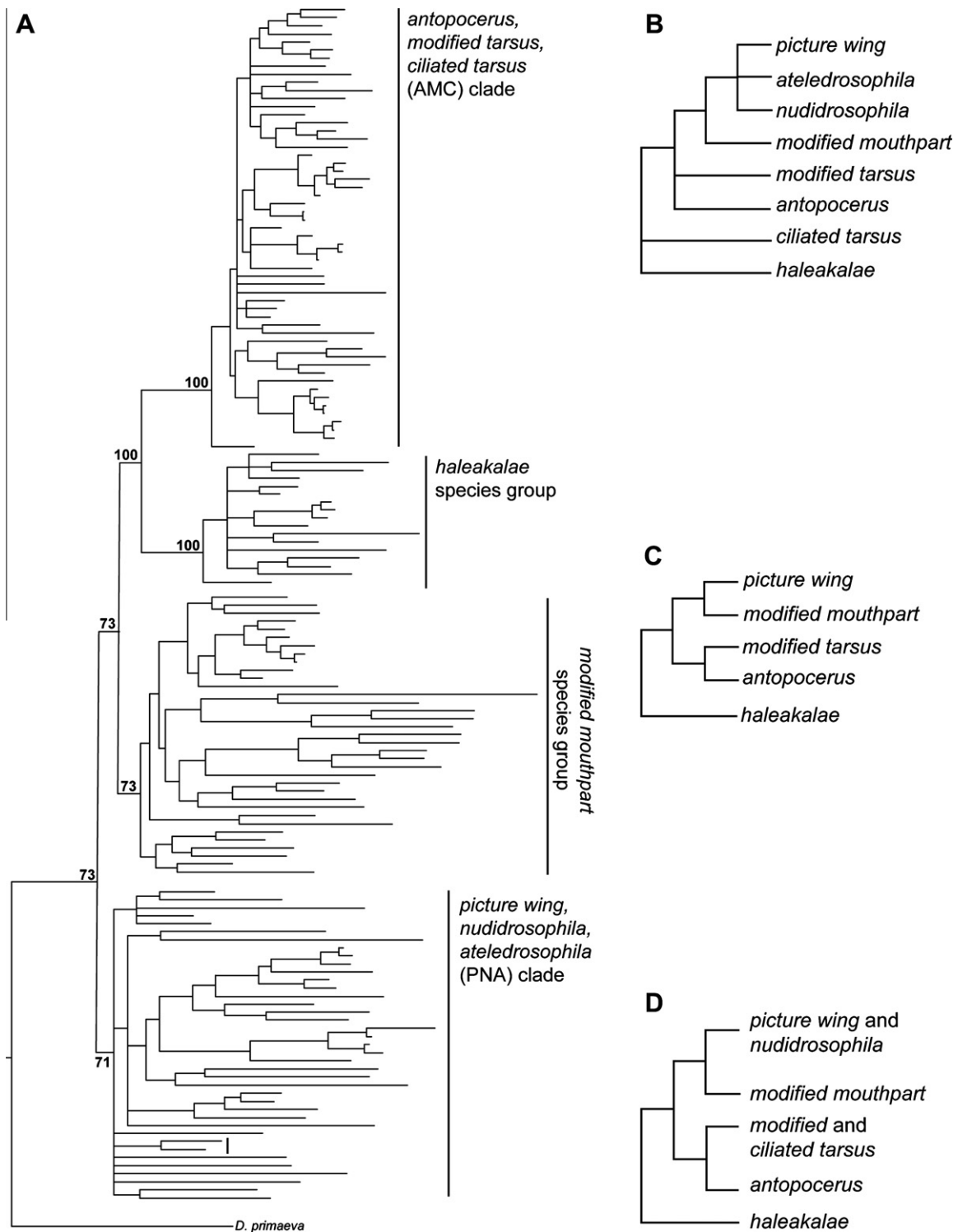


Fig. 3. Summary of phylogenetic relationships among major lineages of Hawaiian *Drosophila*. A. The current study. B. Throckmorton (1966, 1975). C. Composite of Kambysellis et al. (1995) and Baker and DeSalle (1997). D. Bonacum (2001).

Outgroup selection might also play a role in the placement of this group. Previous work used either *D. melanogaster* or a member of the subgenus *Drosophila*, taxa that are quite distantly related to the endemic Hawaiian species, as outgroup taxa. Only a few studies included representatives of the genus *Scaptomyza*, the sister clade of the Hawaiian *Drosophila*, and those that did are characterized by lower support or conflict among the basal branches of the phylogeny (Baker & DeSalle, 1997; Bonacum, 2001; O'Grady and

DeSalle, 2008). More closely related outgroup species not only provide a more rigorous test of monophyly, but may also ameliorate rooting issues seen with more distant outgroup taxa.

It is clear from the present study that additional work is needed to not only refine relationships among species and species subgroups in the various clades of Hawaiian *Drosophila*, but also to resolve relationships at the base of this large radiation. Our novel result of basal *picture wing* and *modified mouthpart* species groups

may be a reflection of the broad taxon sampling and the large number of characters that we have analyzed. In spite of this intriguing result, however, support values at these nodes are not strong and additional analyses must be run with more characters and, if possible, additional taxon sampling. The addition of characters from throughout the nuclear genome will help to resolve the basal branches within this important clade. The phylogenetic and ecological work that we present here yields great insight into how this group has adapted to multiple host plants and types of substrates throughout its evolution.

4.2. Relationships among species groups

Throckmorton (1966) provided names for most of the species groups and subgroups of Hawaiian *Drosophila*, largely based on his analysis of internal morphological characters and male secondary sexual characters. A number of taxonomic studies have elaborated on this structure (Heed, 1968; Kaneshiro, 1976; Kaneshiro, Gillespie, & Carson, 1995). Phylogenetic studies (Bonacum, O'Grady, Kambysellis, & Desalle, 2005; Carson and Stalker, 1968; Kambysellis et al., 1995; O'Grady and Zilversmit, 2004) have

Table 5
Summary of phylogenetic results.

Lineage 1	Lineage 2	Species group	Species subgroup	Total spp. (sampled)	Status (support)	Totals
AMC clade	n/a	<i>Antopocerus</i>	<i>Adunca</i>	2 (2)	M (100)	
			<i>Diamphiodiopoda</i>	6 (2)	M (100)	
			<i>Villosa</i>	7 (0)	–	
				15 (4)	M (100)	<i>Antopocerus</i>
	n/a	<i>Modified tarsus</i>	<i>Bristle tarsus</i>	18 (18 ^a)	P	
			<i>Ciliated tarsus</i>	21 (5)	P	
			<i>Split tarsus</i>	24 (18 ^a)	M (83)	
			<i>Spoon tarsus</i>	12 (10)	P	
			<i>Unplaced</i>	1 (0)	–	
				76 (51)	P	<i>Mod. tarsus</i>
				91 (55 ^a)	M (100)	AMC clade
n/a	n/a	<i>Haleakalae</i>	<i>Anthrax</i>	10		
			<i>Cilifemorata</i>	15		
			<i>Haleakalae</i>	9		
			<i>Luteola</i>	4		
			<i>Polita</i>	10		
			<i>Scitula</i>	5		
			<i>Unplaced</i>	1		
				54 (17)	M (100)	<i>Haleakalae</i>
n/a	n/a	<i>Modified mouthpart</i>	<i>Ceratostoma</i>	4 (1 ^a)	n/a	
			<i>Dissita</i>	14 (3)	P	
			<i>Freycinetiae</i>	9 (1)	n/a	
			<i>Fuscoamoeba</i>	8 (2)	M (100)	
			<i>Hirtitarsus</i>	2 (1 ^a)	n/a	
			<i>Mimica</i>	20 (12 ^a)	P	
			<i>Mitchelli</i>	5 (5)	M (100)	
			<i>Nanella</i>	4 (2)	P	
			<i>Quadrissetae</i>	4 (2 ^a)	P	
			<i>Scolistoma</i>	3 (0)	–	
			<i>Semifuscata</i>	14 (1)	n/a	
			<i>Setiger</i>	4 (2)	P	
			<i>Unplaced</i>	15 (3)	n/a	
				106 (35 ^a)	M (73)	<i>Mod. mpart.</i>
PNA clade	Picture wing clade	<i>Adiastola</i>	<i>Adiastola</i>	12 (1)	n/a	
			<i>Truncipenna</i>	4 (0)	–	
		<i>Anomalipes</i>	n/a	2 (1)	n/a	
		<i>Grimshawi</i>	<i>Conspicua</i>	9 (0)	–	
			<i>Crucigera</i>	8 (5)	M (76)	
			<i>Distingueda</i>	3 (0)	–	
			<i>Hawaiiensis</i>	14 (0)	–	
			<i>Orphnopeza</i>	17 (3)	P	
			<i>Pilimana</i>	5 (2)	M (97)	
			<i>Punalua</i>	8 (1)	n/a	
			<i>Vesciseta</i>	8 (1)	n/a	
			<i>Unplaced</i>	5 (1)	n/a	
				77 (12)	P	<i>Grimshawi</i>
		<i>Planitibia</i>	<i>Cyrtoloma</i>	7 (7)	M (79)	
			<i>Neopicta</i>	3 (3)	M (89)	
			<i>Picticornis</i>	2 (2)	P	
			<i>Planitibia</i>	5 (5)	M (100)	
				17 (17)	P	<i>Planitibia</i>
		<i>Primaeva</i>	n/a	2 (1)	n/a	
				114 (33)	P	pw clade
	n/a	<i>Nudidrosophila</i>	<i>Hirtitibia</i>	4 (3)	M (79)	
			<i>Kahania</i>	2 (0)	–	
			<i>Nudidrosophila</i>	11 (3)	M (99)	
			<i>Okala</i>	5 (0)	–	
			<i>Velata</i>	6 (0)	–	
	n/a	<i>Ateledrosophila</i>	n/a	3 (1)	n/a	
				145 (40)	M (71)	PNA clade

^a Includes a number of new species not included in the total species count.

explicitly tested the monophyly of some lineages and provided a notion of relationships within and among some species groups. Here we present the largest and more taxonomically comprehensive study to date (Table 5) and suggest several changes based on our results (Table 6).

Our results (Fig. 3A) suggest that the *haleakalae* species group is sister to the *modified tarsus* and *antopocerus* species groups. (Throckmorton, 1966, 1975) proposed that the *haleakalae* and *ciliated tarsus* groups to be basal within the Hawaiian *Drosophila*, largely based on their simplistic mating behaviors and reduced sexual dimorphism, particularly in the *haleakalae* group (Fig. 3B). This assertion, however, does not take into account the possibility of complex mating behaviors evolving early in the evolution of the Hawaiian *Drosophila* and then being lost in a secondary event along the branch leading to the *haleakalae* lineage.

With respect to the close relationships between the *antopocerus* and *modified tarsus* species groups, our results are largely congruent with morphology (Throckmorton, 1966, Fig. 3B), previous molecular work (Baker & DeSalle, 1997, Fig. 3C), and ecological associations these species have with leaves of Araliaceae (Heed, 1968). Our results differ in one key point. We find that, in agreement with Bonacum (2001), the *ciliated tarsus* species are part of the *modified tarsus* species group (Fig. 3A and D), not basal in the Hawaiian *Drosophila* (Throckmorton, 1966, Fig. 3B). The types of modifications in the *ciliated tarsus* group, elongate hairs on the foretarsus of males are also seen, albeit to a lesser degree, in the *bristle tarsus* subgroup of the *modified tarsus* species. In fact, the *bristle* and *ciliated tarsus* species are paraphyletic with respect to one another (Fig. 1B), suggesting that bristle length of these sexually selected characters may not be phylogenetically informative. There is no data to support Throckmorton's (1966) placement of the *ciliated tarsus* species group as basal within the Hawaiian *Drosophila*.

Throckmorton considered the picture wing, *ateledrosophila* and *nudidrosophila* species to be closely related, based mainly on the hook-like shape of the distal process of the aedeagus (Fig. 3B). He suggested that the *nudidrosophila* species were sister to the *planitibia* species group, rendering the picture wings paraphyletic (Throckmorton, 1966). With the exception of Bonacum (2001, Fig. 3D) previous molecular studies have not sampled *nudidroso-*

phila and no study to date has sampled *ateledrosophila*. We include multiple representatives of these lineages and our results are in agreement with Throckmorton's (1966) placement. Our results suggest that the *picture wing* species are not monophyletic (Fig. 1A). This result is not surprising given that patterned wings have evolved multiple times in the Drosophilidae (Markow & O'Grady, 2006) and even within other lineages of Hawaiian *Drosophila* (e.g., the *fuscoamoeba* subgroup of the modified mouthparts species group (Magnacca and O'Grady, 2008).

The basal-most lineages in our phylogeny are the PNA clade, a lineage containing the picture wing, *nudidrosophila* and *ateledrosophila* species groups and the *modified mouthpart* species group (Fig. 3A). While this runs counter to previous work, which placed them as sister taxa and derived within the Hawaiian *Drosophila* (Baker & DeSalle, 1997; Kambysellis et al., 1995; Throckmorton, 1966, Fig. 3B–D), these two are the largest and oldest groups and most likely represent early divergences within this clade.

4.3. Relationships within species groups

Few studies have examined the species level relationships within the various species groups of Hawaiian *Drosophila*. The *picture wing* species group has received the most attention, largely due to the efforts of Hamp Carson and colleagues (reviewed in Kaneshiro et al., 1995). They provide detailed hypotheses of relationships among subgroups of the picture wings based on polytene chromosome banding patterns, although they did not sample the related *ateledrosophila* or *nudidrosophila* species. O'Grady and Zilversmit (2004) produced a phylogeny and proposed relationships among subgroups of the *haleakalae* species group. There are no hypotheses of relationships within the *nudidrosophila*, *ateledrosophila*, and *modified mouthpart* species groups or within the AMC clade.

4.3.1. The picture wing species (PNA clade, in part)

Carson spent over 40 years studying the phylogenetic relationships among the picture wing species (reviewed in Kaneshiro et al., 1995; Markow & O'Grady, 2006). This body of research found that the *grimshawi*, *adiastola* and *planitibia* species groups (*sensu* Throckmorton, 1966) were monophyletic and designated them as clades. Relationships within each clade, however, were less clear.

Table 6
Proposed Taxonomic Changes.

Lineage	Previous work	Current study
PNA clade	Not addressed	Include all picture wing, <i>nudidrosophila</i> and <i>ateledrosophila</i> species in this clade
<i>Planitibia</i> subgp	<i>Planitibia</i> , <i>neopicta</i> , <i>cyrtoloma</i> , and <i>picticornis</i> species groups	Remove <i>picticornis</i> subgroup from the <i>planitibia</i> species group
<i>Picticornis</i> subgp	<i>D. picticornis</i> , <i>D. setosifrons</i>	Support for the paraphyly of this subgroup is not strong, retain pending further research
<i>Dissita</i> subgp	14 Taxa (Magnacca and O'Grady, 2008)	Include unplaced taxon <i>D. barbata</i>
<i>Mimica</i> subgp	20 species included (O'Grady et al., 2003; Magnacca and O'Grady, 2006, 2008)	Support for inclusion of a new species in the <i>hirtitarsus</i> subgroup, retain current configuration pending additional sampling
<i>Nanella</i> subgp	4 Taxa included (Magnacca and O'Grady, 2008)	This subgroup is clearly paraphyletic with respect to the part of the <i>quadrisetae</i> subgroup, the taxonomic status of both subgroups should be shifted to "unplaced" pending further sampling
<i>Quadrisetae</i> subgp	4 Taxa included (Magnacca and O'Grady, 2008)	This subgroup is paraphyletic and its taxonomic status should be shifted to "unplaced" pending further sampling
<i>Setiger</i> subgp	4 Taxa included (Magnacca and O'Grady, 2008)	Include currently unplaced species <i>D. tetraspilota</i>
<i>Mod.tarsus</i> subgp	76 Taxa included (O'Grady et al., 2010)	The <i>antopocerus</i> species group is imbedded within the <i>modified tarsus</i> species group. We are elevating all <i>modified tarsus</i> subgroups (<i>bristle</i> , <i>ciliated</i> , <i>split</i> , <i>spoon</i>) to species group rank and eliminating "modified tarsus" as a formal group
<i>Bristle tarsus</i> subgp	18 Species included (O'Grady et al., 2010), with >10 new taxa to be described	Our analyses support the notion that these species are paraphyletic with respect to the <i>ciliated tarsus</i> species group. Further research is needed to specifically address these issues
<i>Ciliated tarsus</i> subgp	21 Species included (O'Grady et al., 2010), with >20 more awaiting description	This group is paraphyletic with respect to the <i>bristle tarsus</i> species group. Additional work will be needed before the taxonomic relationships between these two groups can be resolved
<i>Spoon tarsus</i> subgp	12 Species included (Lapoint, Magnacca, & O'Grady, 2009)	We formally remove <i>D. atroscutellata</i> and <i>D. fastigata</i> from this group and place them <i>incertae sedis</i> , pending further work

The *grimshawi* clade is divided into two groups, *grimshawi* and *glabriapex*. The *grimshawi* group is monophyletic, but the *glabriapex* group is resolved as a basal paraphyly. Within the *grimshawi* group, only the *hawaiiensis* subgroup is monophyletic; the *orphnopeza* and *grimshawi* subgroups are paraphyletic with respect to one another (Kaneshiro et al., 1995). Likewise in the *glabriapex* species group, only the *punalua* and *distingueda* subgroups are monophyletic. The *glabriapex*, *conspicua* and *vesciseta* subgroups are all paraphyletic (Kaneshiro et al., 1995). The two subgroups in the *adiastola* clade, *adiastola* and *truncipennis*, are also paraphyletic (Kaneshiro et al., 1995). The *planitibia* clade is better resolved at the subgroup level, with the *planitibia*, *neopicta* and *picticornis* subgroups recovered as monophyletic. Only the *cyrtoloma* subgroup is paraphyletic (Kaneshiro et al., 1995).

The widespread paraphyly within the picture wing species groups and subgroups is probably more a restriction of using relatively rare events like chromosomal inversions to track phylogeny in a rapidly evolving group than problems with each group (O'Grady et al., 2002). Recent molecular work on the *planitibia* clade (Bonacum, O'Grady, Kambysellis, & Desalle, 2005) has shown all of the component subgroups to be monophyletic. Although taxon sampling was not explicitly designed to test relationships at the species and species subgroup levels within the *picture wing* clade, the current study suggests several species groups and subgroups within this larger clade are monophyletic. Within the *grimshawi* species group, for example, the *crucigera* and *pilimana* subgroups are both monophyletic. Likewise, three of the four subgroups of the *planitibia* species group (*planitibia*, *neopicta*, *cyrtoloma*) are monophyletic. Only the *picticornis* subgroup is paraphyletic, suggesting that a revision of the taxonomic status of this subgroup may be in order. Additional taxon sampling, specifically targeting species relationships within the picture wings, will be needed to more rigorously test monophyly of these groups.

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