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# Evolution and biogeography of native Hawaiian *Hylaeus* bees (Hymenoptera: Colletidae)

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**Abstract**

The only bees native to the Hawaiian Islands form a single clade of 60 species in the genus *Hylaeus*. The group is understudied and relatively poorly known. A data set consisting of 1201 base pairs of the mitochondrial genes cytochrome oxidase I and II and tRNA-Leucine, and 14 morphological characters was used to construct a phylogenetic tree for 48 of the 60 known species. Genetic variation was high, including amino acid changes, and a number of species showed evidence of heteroplasmy. Tree support was low due to high levels of homoplasy. Biogeographical analysis using DIVA indicates that early radiation took place on the island of Hawaii. This places an upper age limit of only 0.4–0.7 Myr for the group, an unusually short time for such a large radiation. Moreover, it is an unusual biogeographical pattern among the Hawaiian biota.



The fauna and flora of the Hawaiian Islands are characterized by a small number of introductions followed by extensive speciation within the archipelago (Zimmerman, 1948). The result is a native biota that is depauperate at the order, family, and genus level, but with high species diversity, high levels of endemism, and many extraordinarily large radiations. The Hymenoptera are a prime example of such imbalance. The suborder Symphyta, with 14 families and 8000 species worldwide (Goulet and Huber, 1993), is absent from the native fauna. The Aculeata clade (29 families, about 50 000 described species; Goulet and Huber, 1993) is represented by about 400 species in only four families derived from seven introductions: two Bethylidae, two Crabronidae, two Vespidae, and one Colletidae (Nishida, 2002). Three of these—*Sierola* (Bethylidae, 184 species plus many more undescribed), the "*Nesodynerus*" group of *Odynerus* (Vespidae, 112 species), and *Hylaeus* (Colletidae, 60 species)—account for 90% of the species. The *Hylaeus* are the only bees native to the Hawaiian Islands.

The Hawaiian *Hylaeus* belong to the subgenus *Nesoprosopis*, which is otherwise primarily found in Japan. One species, *H. pectoralis*, extends across to Europe, and undescribed species have been collected from China (Hirashima, 1977; Ikudome, 1989). In Hawaii, the group has evolved from a single introduction to at least 60 species (Daly and Magnacca, 2003), more than the total number of *Hylaeus* in America north of Mexico (55 species; Snelling, 1966). The Hawaiian radiation makes the otherwise-minor *Nesoprosopis* the largest subgenus of *Hylaeus* aside from *Hylaeus sensu strictu* (Michener, 2000; the Australian *Prosopisteron* also has more species but is polyphyletic, T. Houston, pers. comm.). As with many endemic insect groups, the biology of *Hylaeus* in Hawaii is largely unknown. A taxonomic revision was recently completed (Daly and Magnacca, 2003), but little is known of the bees' evolution (phylogenetic relationships, biogeographical history), habitat requirements (nesting behavior, pollen usage), or conservation biology (competitive pressure from exotic bees, usage of introduced plants). Most of what is known is based on scattered observations of a few specimens of a single species (Williams, 1927; Swezey, 1954; Daly and Coville, 1982). The Hawaiian radiation includes the only cleptoparasitic colletids

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(Michener, 2000) and a large number of ground-nesting species, a relatively uncommon habit for *Hylaeus* (Daly and Magnacca, 2003), so there is much fertile ground for future research.

Hawaii has recently become a focus of biogeographical work (Wagner and Funk, 1995). The Hawaiian insect fauna contains a number of spectacular radiations that are well-suited to such study. While the *Drosophila* are best known, with probably over 1000 species, there are several genera with over 100 representatives in the islands, and many with over 50 (Liebherr, 2001). Some, such as *Lispocephala* (Muscidae) and *Sierola* (Bethylidae), are widespread in continental areas but the number of species in Hawaii far exceeds those in the rest of the world combined (Evans, 1978; Hardy, 1981). In many others, such as Drosophilidae, Nitidulidae and Hylaeinae, the Hawaiian species form a significant proportion of the world fauna.

As a linear hot-spot volcanic chain, the geological history of Hawaii is well-known: as new volcanoes form to the south-east, the islands to the north-west erode, subside, and eventually disappear (Carson and Clague, 1995). Because the ages of the islands are known from K-Ar dating of volcanic rock, biogeographical conclusions can be used to infer the approximate arrival time of a group's progenitor. Phylogenetic studies of groups that arrived at or before the emergence of the oldest current high island, Kauai, usually find species from older islands to be basal, and those on younger islands apical (Wagner and Funk, 1995). This reflects a pattern of dispersal on to new islands as they arise, followed by within-island radiation that is dependent on time, habitat heterogeneity, and dispersal ability. More recent arrivals tend to show a more random pattern, reflecting the availability of multiple islands as targets for dispersal (Lowrey, 1995).

The basic biogeographical questions in an isolated island-chain setting are: (1) when did the progenitor of a group arrive; (2) where did it first become established; and (3) how did diversification progress geographically? Once a phylogeny is established for the species, the last two questions can be answered using biogeographical methods such as component analysis (Page, 1994) and DIVA (Ronquist, 2001). The question of time of arrival is the most difficult to answer, but it is the most interesting in terms of its implications for the evolution of the group. For example, clades of five species that are 3 million years (Myr) old are not unusual; whereas 100-species radiations that are only 1 Myr old are rare. The latter case indicates that strong evolutionary pressures are or were being exerted to cause rapid diversification.

In an attempt to answer these questions for the Hawaiian *Hylaeus*, we developed a phylogeny based on DNA sequences and morphology. The relatively recent origin of the group, and the generally low level of morphological diversity in *Hylaeus* in general, make genetic methods more productive for phylogenetics in the group. The mitochondrial genes cytochrome oxidase I and II were chosen based on their rate of evolution: they are generally considered to be the most conservative of the mitochondrial genes, but still change more rapidly than most nuclear genes (Simon et al., 1994). This relatively fast rate of evolution has made these genes useful in estimating species or species-group level phylogenies of other Hawaiian groups (Wagner and Funk, 1995; C. Ewing, unpubl. data) and other bees (Danforth, 1999). Several nuclear genes (EF-1a, *wingless*, arginine kinase and opsin), sequenced for a subset of species, had too little variation to be informative (unpubl. data).

## Materials and methods

### *Taxon sampling*

In an effort to make the phylogeny as complete as possible, we attempted to include all species of Hawaiian *Hylaeus*. Forty-nine of the 60 described species were obtained for sequencing. One of these, *H. akoko*, was not included due to a high level of polymorphisms (see below under Results). Nearly all specimens were collected during the period 1999–2002. All major islands were visited, and individuals from all available island populations were included in the data set unless their sequences differed by less than five bases (including multiple genotypes from one island; see Results). Most bees were collected by hand net over flowers; a few were caught in pan traps. A large set of outgroups spanning the genus was used, including (from nearest to farthest) two Japanese *Nesoprosopis*, 13 Holarctic *Hylaeus* representing six subgenera, and four Australian *Hylaeus* representing three subgenera. Species and collecting sites are shown in Table 1.

### *DNA extraction*

Total DNA was extracted using standard protocols (Doyle and Doyle, 1990). Tissue was taken from the thoracic musculature, reproductive organs or legs depending on the rarity of available specimens. Samples were macerated in individual 1.5 mL Eppendorf tubes with 2 × CTAB extraction buffer and 100 mg Proteinase K. Samples were incubated for 2 h at 55 °C, extracted with 24 : 1 chloroform–isoamyl alcohol, digested for 30 min with 10 mg RNase, and extracted again with 25 : 24 : 1 phenol–chloroform–isoamyl alcohol and 24 : 1 chloroform–isoamyl alcohol. DNA was precipitated with 2.5 volumes of ice-cold 100% ethanol and 0.1 volume 3 M sodium acetate, washed with 80% ethanol, and resuspended in 50 mL Tris–EDTA (pH 7.6) buffer.

Table 1
Specimens used in sequencing. All are members of the genus *Hylaeus*; subgeneric names are used here and in trees. Abbreviations are HAVO (Hawaii Volcanoes National Park), HALE (Haleakala National Park), NWR (National Wildlife Refuge), PTA (Pohakuloa Training Area), FR (state Forest Reserve) and NAR (state Natural Area Reserve). Specimens without a GenBank accession number were not used in phylogenetic analysis due to similarity to the preceding specimen. Multiple GenBank numbers indicate different clones from a single individual

| Species | State/Island | Locality | Date | Collector | GenBank no. |
|---|---|---|---|---|---|
| Australian outgroups | | | | | |
| *Euprosopis elegans* | S. Australia | 10 km E Kimba | 5 Jan 1999 | B. Danforth | AY913953 |
| *Gnathoprosopis albonitens* | Hawaii | Kapoho 1960 flow | 16 Jan 1999 | K. Magnacca | AY913954 |
| *Gnathoprosopis amiculus* | S. Australia | 10 km E Kimba | 5 Jan 1999 | B. Danforth | AY913955 |
| *Rhodohylaeus proximus* | S. Australia | 10 km E Kimba | 5 Jan 1999 | B. Danforth | AY913956 |
| Palearctic outgroups | | | | | |
| *Cephalylaeus basalis* | California | Jackson Meadow | 27 Jun 2000 | J. Ascher | AY913957 |
| *Cephalylaeus nunemacheri* | California | San Antonio Junction | 28 May 1999 | J. Ascher | AY913958 |
| *Hylaeus ellipticus* | New York | Ithaca | 22 May 1999 | J. Ascher | AY913960 |
| *Hylaeus leptocephalus* | New York | Cornell University | 27 Jul 1999 | J. Ascher | AY913959 |
| *Hylaeus mesillae* | New York | Ithaca | 22 May 1999 | J. Ascher | AY913961 |
| *Paraprosopis calvus* | California | San Antonio Junction | 28 May 1999 | J. Ascher | AY913962 |
| *Paraprosopis wootoni* | Arizona | Chiracahua Mt., Onion Pass | 20 Sep 1999 | K. Magnacca | AY913963 |
| *Prosopella hurdi* | Arizona | 7.4 mi. NW Portal | 19 Sep 1999 | K. Magnacca | AY913964 |
| *Prosopis affinis* | New York | Ithaca | 22 May 1999 | J. Ascher | AY913965 |
| *Prosopis episcopalis* | California | San Antonio Junction | 28 May 1999 | J. Ascher | AY913966 |
| *Prosopis modestus* | New York | Ithaca | 22 May 1999 | J. Ascher | AY913967 |
| *Spatulariella hyalinatus* | New York | Cornell University | 7 Jul 1999 | J. Ascher | AY913968 |
| *Spatulariella punctatus* | California | U.C. Berkeley campus | 21 Jun 1999 | J. Ascher | AY913969 |
| Japanese *Nesoprosopis* | | | | | |
| *Nesoprosopis globula* | Japan | Inohara-kogen Yokota-cho | 10 Oct 1999 | Y. Maeta | AY913970 |
| *Nesoprosopis insularum* | Japan | Kuji Setouchi-cho | 27 Mar 1999 | Y. Maeta | AY913971 |
| Hawaiian *Nesoprosopis* | | | | | |
| *Nesoprosopis andrenoides* | Kauai | Alakai Swamp Trail | 21 Aug 1999 | K. Magnacca | AY913972 |
| *Nesoprosopis angustulus* | Maui | Makawao Forest Reserve | 10 Aug 2002 | K. Magnacca | AY913973 |
| *Nesoprosopis angustulus* | Molokai | West Kawela Gulch | 2 Jun 2001 | K. Magnacca | AY914036 |
| *Nesoprosopis anthracinus* | Hawaii | South Point | 18 Jul 1999 | K. Magnacca | AY913974 |
| *Nesoprosopis anthracinus* | Maui | Manawainui west | 23 Jun 1999 | K. Magnacca | AY913975 |
| *Nesoprosopis anthracinus* | Oahu | Kaena Point NAR | 12 Jun 1999 | K. Magnacca | AY913976 |
| *Nesoprosopis anthracinus* | Molokai | Moomomi Preserve | 26 Jun 1999 | K. Magnacca | |
| *Nesoprosopis assimulans* | Kahoolawe | Kamohio | 17 Feb 1997 | D. Foote | AY913977 |
| *Nesoprosopis assimulans* | Maui | Lahainaluna | 3 Aug 1999 | K. Magnacca | AY913978 |
| *Nesoprosopis assimulans* | Lanai | Polihua Rd. | 17 Jun 1999 | K. Magnacca | |
| *Nesoprosopis chlorostictus* | Kauai | Kokee Rd. | 24 Aug 1999 | K. Magnacca | AY913979 |
| *Nesoprosopis coniceps* | Hawaii | Saddle Rd. | 10 Jul 1999 | K. Magnacca | AY913980 |
| *Nesoprosopis coniceps* | Maui | HALE, Koolau Gap | 6 Aug 1999 | K. Magnacca | AY913981 |
| *Nesoprosopis connectens* | Hawaii | HAVO, Kipuka Nene | 28 Jun 1998 | K. Magnacca | AY913982 |
| *Nesoprosopis connectens* | Maui | Waihee Ridge Trail | 5 Aug 1999 | K. Magnacca | AY913983 |
| *Nesoprosopis connectens* | Oahu | Wiliwilinui Trail | 26 Jul 1999 | K. Magnacca | |
| *Nesoprosopis connectens* | Kauai | Polihale State Park | 25 Aug 1999 | K. Magnacca | AY913984 |
| *Nesoprosopis crabronoides* | Hawaii | HAVO, Olaa Small Tract | 30 July 2000 | K. Magnacca | AY913985 |
| *Nesoprosopis crabronoides* | Hawaii | Kona Forest Unit, Hakalau NWR | 3 August 2000 | K. Magnacca | AY913986 |
| *Nesoprosopis difficilis* | Hawaii | HAVO, Mauna Loa Road | 2 January 1999 | K. Magnacca | AY913987 |
| *Nesoprosopis difficilis* | Maui | Waikamoi Preserve | 4 August 1999 | K. Magnacca | AY913988 |
| *Nesoprosopis difficilis* | Lanai | Munro Trail | 7 August 1999 | K. Magnacca | |
| *Nesoprosopis difficilis* | Molokai | Puu Kolekole | 28 June 1999 | K. Magnacca | AY913989 |
| *Nesoprosopis dimidiatus* | Hawaii | PTA, Kipuka Alala | 14 July 1999 | K. Magnacca | AY913990 AY913991 |
| *Nesoprosopis dumetorum* | Hawaii | Tree Planting Road | 5 January 1999 | K. Magnacca | AY913992 |
| *Nesoprosopis facilis* | Molokai | Alau, Kalaupapa NHP | 31 August 2005 | K. Magnacca | DQ492297 |
| *Nesoprosopis filicum* | Hawaii | Kona Forest Unit, Hakalau NWR | 3 August 2000 | K. Magnacca | AY913993 |
| *Nesoprosopis flavifrons* | Kauai | Polihale State Park | 25 August 1999 | K. Magnacca | AY913994 |
| *Nesoprosopis flavifrons* | Lehua | West Horn | 19 February 2002 | K. Wood | AY913995 |
| *Nesoprosopis flavipes* | Hawaii | HAVO, Kipuka Nene | 3 January 1999 | K. Magnacca | AY913996 |
| *Nesoprosopis flavipes* | Hawaii | Hale Pohaku | 10 July 1999 | K. Magnacca | AY913997 |
| *Nesoprosopis flavipes* | Lanai | Kahue | 7 August 1999 | K. Magnacca | AY913998 |
| *Nesoprosopis fuscipennis* | Maui | Kahoma | 22 May 2001 | K. Magnacca | AY914002 AY914003 |
| *Nesoprosopis fuscipennis* | Lanai | Munro Trail | 16 June 1999 | K. Magnacca | AY913999 |
| *Nesoprosopis fuscipennis* | Molokai | Kamakou Road | 27 June 1999 | K. Magnacca | AY914000 AY914001 |
| *Nesoprosopis haleakalae* | Maui | Puu Kukui Tr. 4500 m | 8 August 2000 | K. Magnacca | AY914004 |

Table 1
*Continued*

| Species | State/Island | Locality | Date | Collector | GenBank no. |
|---|---|---|---|---|---|
| *Nesoprosopis haleakalae* | Molokai | West Kawela Gulch | 28 June 1999 | K. Magnacca | AY914005 |
| *Nesoprosopis hilaris* | Molokai | Moomomi Preserve | 30 June 1999 | K. Magnacca | AY914006 |
| *Nesoprosopis hirsutulus* | Kauai | Alakai, 1.5 mi. NW Keaku | 2 November 1999 | D. Hopper | AY914007 |
| *Nesoprosopis hostilis* | Kauai | Polihale State Park | 25 August 1999 | K. Magnacca | AY914008 |
| *Nesoprosopis hula* | Hawaii | HAVO, Tree Molds | 10 August 1999 | K. Magnacca | AY914009 AY914010 |
| *Nesoprosopis inquilina* | Hawaii | HAVO, 0.9 mi. S Mauna Loa Road | 4 January 1999 | K. Magnacca | AY914012 |
| *Nesoprosopis kauaiensis* | Kauai | Alakai Swamp Trail | 3 July 1999 | K. Magnacca | AY914013 |
| *Nesoprosopis kokeensis* | Kauai | Kokee Road | 24 August 1999 | K. Magnacca | AY914014 |
| *Nesoprosopis kona* | Hawaii | PTA, Kipuka Alala | 14 July 1999 | K. Magnacca | AY914015 |
| *Nesoprosopis kuakea* | Oahu | Moho Gulch Ridge | 1 August 1997 | D. Hopper | AY914016 AY914017 |
| *Nesoprosopis kukui* | Hawaii | HAVO, Kahuku 3600′ | 12 July 2005 | K. Magnacca | DQ492298 |
| *Nesoprosopis kukui* | Maui | Puu Kukui Tr. 7000 m | 11 August 2000 | K. Magnacca | AY914018 |
| *Nesoprosopis laetus* | Hawaii | HAVO, Kipuka Nene | 3 January 1999 | K. Magnacca | AY914019 |
| *Nesoprosopis laetus* | Maui | Lahainaluna | 3 August 1999 | K. Magnacca | AY914021 |
| *Nesoprosopis laetus* | Lanai | Kanepuu Preserve, Kahue Unit | 15 June 1999 | K. Magnacca | |
| *Nesoprosopis laetus* | Oahu | Pahole NAR | 8 June 2002 | K. Magnacca | |
| *Nesoprosopis laetus* | Kauai | Nualolo Trail | 19 January 1999 | K. Magnacca | AY914020 |
| *Nesoprosopis longiceps* | Maui | Waiehu dune | 5 August 1999 | K. Magnacca | AY914022 |
| *Nesoprosopis longiceps* | Lanai | Polihua Road | 17 June 1999 | K. Magnacca | |
| *Nesoprosopis longiceps* | Molokai | Moomomi Preserve | 26 June 1999 | K. Magnacca | |
| *Nesoprosopis longiceps* | Oahu | Kaena Point NAR | 12 June 1999 | K. Magnacca | AY914023 |
| *Nesoprosopis mana* | Oahu | Manana Trail | 3 March 2002 | K. Magnacca | AY914024 |
| *Nesoprosopis mimicus* | Oahu | Wiliwilinui Trail | 26 July 1999 | K. Magnacca | AY914025 |
| *Nesoprosopis muranus* | Hawaii | Volcano Village | 5 August 2000 | K. Magnacca | AY914011 |
| *Nesoprosopis mutatus* | Kauai | Kahuamaa Flat | 24 August 1999 | K. Magnacca | AY914026 |
| *Nesoprosopis nivicola* | Maui | HALE, Halemauu Trail | 19 June 1999 | K. Magnacca | AY914027 |
| *Nesoprosopis ombrias* | Hawaii | South Point | 17 July 1999 | K. Magnacca | AY914028 |
| *Nesoprosopis paradoxicus* | Hawaii | PTA, Kipuka Alala | 14 July 1999 | K. Magnacca | AY914030 |
| *Nesoprosopis pele* | Hawaii | PTA, Kipuka Alala | 14 July 1999 | K. Magnacca | AY914031 |
| *Nesoprosopis psammobius* | Maui | Eleilei Bay | 8 August 2002 | K. Magnacca | AY914032 |
| *Nesoprosopis pubescens* | Hawaii | HAVO, Devastation Trail | 8 January 1999 | K. Magnacca | AY914033 |
| *Nesoprosopis rugulosus* | Hawaii | HAVO, Kipuka Nene | 16 May 1998 | K. Magnacca | AY914029 |
| *Nesoprosopis setosifrons* | Hawaii | HAVO, Tree Molds | 9 August 1999 | K. Magnacca | AY914034 |
| *Nesoprosopis solaris* | Kauai | Polihale State Park | 25 August 1999 | K. Magnacca | AY914035 |
| *Nesoprosopis specularis* | Hawaii | Kona Forest Unit, Hakalau NWR | 8 July 1999 | K. Magnacca | AY914037 |
| *Nesoprosopis specularis* | Molokai | West Kawela Gulch | 29 August 2005 | K. Magnacca | DQ492299 |
| *Nesoprosopis specularis* | Oahu | Manana Trail | 19 February 2002 | K. Magnacca | AY914040 |
| *Nesoprosopis specularis* | Kauai | Na Pali-Kona FR | 4 July 1999 | K. Magnacca | AY914039 |
| *Nesoprosopis specularis* | Kauai | Awaawapuhi Trail | 4 July 2000 | K. Magnacca | AY914038 |
| *Nesoprosopis sphecodoides* | Hawaii | HAVO, Kipuka Nene | 11 July 1999 | K. Magnacca | AY914041 |
| *Nesoprosopis takumiae* | Maui | HALE, Kilohana Pali | 27 April 1999 | R. Takumi | AY914042 |
| *Nesoprosopis unicus* | Maui | Puu Kukui Tr. 4500 m | 8 August 2000 | K. Magnacca | AY914046 |
| *Nesoprosopis unicus* | Molokai | Kamakou Road | 27 June 1999 | K. Magnacca | AY914044 AY914045 |
| *Nesoprosopis unicus* | Oahu | Wiliwilinui Trail | 13 June 1999 | K. Magnacca | AY914043 |
| *Nesoprosopis volatilis* | Maui | HALE, Halemauu Trail | 23 June 1999 | K. Magnacca | AY914049 |
| *Nesoprosopis volcanicus* | Hawaii | HAVO, Mauna Loa Road | 2 January 1999 | K. Magnacca | AY914047 |
| *Nesoprosopis volcanicus* | Maui | HALE, Kaupo Trail | 28 June 2000 | K. Magnacca | AY914048 |

### *PCR and sequencing*

PCR products were generated as two fragments using the primers C1-J-2183 (''Jerry'') and TL2-N-3014 (''Pat'') and a modified version of C2-N-3389 (''Marilyn II''; sequence 5′-CATATCTTCARTATCCATTGATGT-CC-3′) (Simon et al., 1994), and a new forward primer (5′-TTTCTWGGITTAATRGGWATRCC-3′), which is called C1-J-2777 under Simon et al.'s (1994) naming system. For some specimens a single fragment was obtained using Jerry and Marilyn II. This spans portions of both COI and COII, and the intervening tRNA-Leucine. PCR reactions were run under the following program: an initial 94° denaturation for 60 s, followed by 35 cycles of denaturation at 94° for 30 s, annealing at 52° for 60 s, and extension at 72° for 60 s. An extension time of 75 s was used when amplifying the entire fragment at once. Gel purification was unnecessary as all samples produced a single product. PCR products were directly purified using the Promega Wizard PCR Preps DNA Purification Kit (Promega Corp., Madison, WI). Sequencing was performed on an ABI 377 automated sequencer

through the Cornell Evolutionary Genetics Core Facility using the BigDye system.

The PCR primers were used for sequencing. Chromatograms were edited and sequences assembled in Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI), and aligned in MegAlign (DNAStar, Madison, WI). The total length of the fragment obtained was 1195–1201 bp for ingroup taxa, including 822 bp of the 3′ end of COI, a small non-coding region, tRNA-Leucine, and 300 bp of the 5′ end of COII. Greater variation in the length of the non-coding region resulted in outgroup sequences ranging from 1187 to 1218 bp. Alignment of coding regions and the tRNA was largely trivial. Alignment of the non-coding region was uncertain for many ingroup species, and impossible among the outgroups. This section was removed for analysis, leaving 1201 aligned bp (including gaps in the tRNA) in the analysis.

In most cases, sequences were completely unambiguous. However, the sequences of 10 species—*H. akoko*, *H. dimidiatus*, *H. fuscipennis*, *H. hula*, *H. kokeensis*, *H. kuakea*, *H. kukui*, *H. mimicus*, *H. pubescens* and *H. unicus*—had significant numbers of polymorphisms. These appeared in repeated sequencing of an extraction and in multiple individuals of the same species, ruling out contamination. For these species, cloning (pGEM-T Easy Vector System, Promega) was carried out to obtain clean sequences.

### *Morphological data*

Fourteen morphological characters were included (see Appendix 1). All character states were taken from direct observations of recently collected specimens. Some rare species could not be dissected and are marked unknown (missing data) for characters that require it (e.g., female labial fovea). The female of *H. kuakea* is unknown, and it is marked as missing data for female-specific characters.

### *Cladistic analysis*

All analyses were performed using PAUP* 4.0b10. The complete matrix (DNA and morphological data) was analyzed under parsimony. Searches were conducted with 1000 replicates, holding a maximum of 30 trees at each step, followed by searching with the option "search all trees in memory" (equivalent to "h/30 mu*1000 max*" in NONA). Support was estimated using bootstrap (200 replicates) and Bremer support. Constraint trees for Bremer support analysis were constructed using AutoDecay 5.0 (Eriksson, 2003).

### *Biogeographical analysis*

In order to identify the locations of origin and diversification of the Hawaiian *Hylaeus*, biogeography was analyzed using DIVA v1.2 (Ronquist, 2001). This program gives geographic locations for common ancestors using a process similar to character optimization. There are several practical reasons for choosing dispersal–vicariance analysis (Ronquist, 1997) over reconciled tree/component analysis (Page, 1994). First, the primary question is to determine where species-group origin, divergence, and diversification took place, rather than to ascertain the history of the area based on the species that occupy it. The latter is already well-known because of the relatively simple geological history of the Hawaiian Islands (Carson and Clague, 1995). Second, most of the islands were never connected; therefore, the primary method of separation is dispersal to a new island rather than vicariance (the islands of Maui Nui were joined during glacial maxima (Price and Elliott-Fisk, 2004), but the near-absence of endemic species on the smaller islands suggests that vicariance has not been a factor in speciation of *Hylaeus*). A large number of dispersals results in an extremely complex reconciled tree with many duplications (Ronquist, 1997). This problem is magnified by the high dispersal ability of *Hylaeus* (inferred from the number of multi-island species), which may result in sister species that do not inhabit neighboring islands. Finally, when starting from a cladogram with a complex, semirandom pattern of dispersals, the general area cladogram produced by component analysis is strongly influenced by a few clades that exhibit similar patterns but do not reflect the overall pattern of the group.

Five areas were used in the analysis: Hawaii, Maui + Lanai, Molokai, Oahu and Kauai (including Niihau, the small islet of Lehua, and Nihoa) (Fig. 1). The grouping of Maui and Lanai was done because the latter has no endemic species, and specimens collected there possessed mtDNA sequences identical or nearly identical to those from Maui. In contrast, individuals from Molokai almost always had sequences different from Maui specimens. For purposes of clarity, as most Oahu species are also found throughout Maui Nui, they are depicted as one area in the figures.

The output from DIVA gives the distribution at tree nodes, not along the branches. As a result, the ancestor of sister species from Maui and Hawaii will be shown as being on both islands, even if it spent most of its evolutionary history on Hawaii and then dispersed to Maui, with subsequent divergence into two species. In preparing the trees here (Figs 4 and 5), the results are applied to the branches based on the previous ancestor. For the example above, if the previous node was indicated as being only Hawaii, the branch leading to the Hawaii/Maui species pair is marked as Hawaii, as there is little doubt that the ancestor designated by that branch lived on Hawaii before dispersal to Maui led to speciation. If the

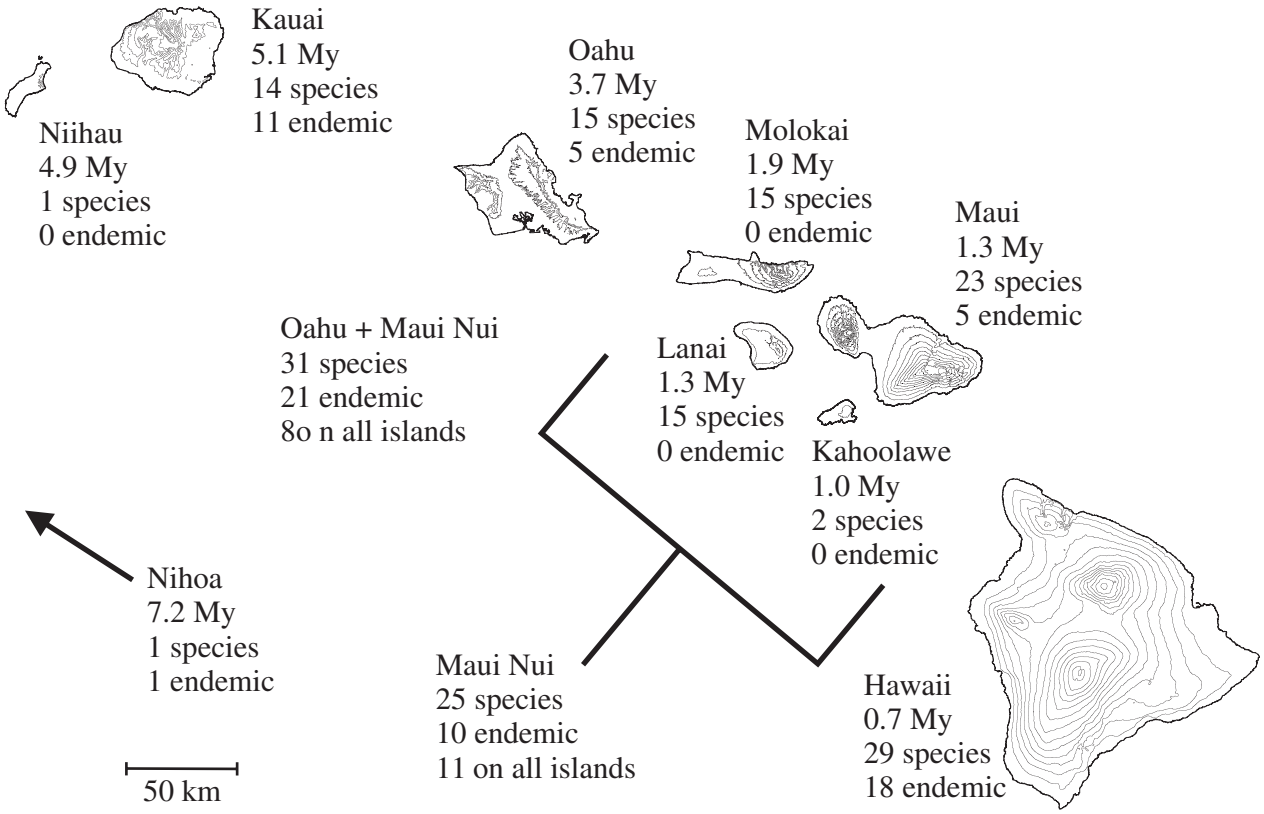


Fig. 1. The main islands of the Hawaiian chain, with 300 m contours. Under the name of each island is its approximate age in millions of years (Moore and Clague, 1992; Carson and Clague, 1995). Species numbers for "all islands" of Maui Nui do not include Kahoolawe, which has a depauperate fauna.

previous node came out as Hawaii/Maui, the branch is marked ambiguous, as the ancestor could have lived on Hawaii, Maui, or both.

As DIVA requires fully resolved trees, all most-parsimonious trees were analyzed. All taxa were retained for the analyses (i.e., the full tree, Fig. 2, was used rather than the simplified tree shown in Fig. 4), with the exception of *H. fuscipennis* and *H. unicus*. These species both occur on multiple islands, and have heteroplasmy in their mtDNA sequences (see below). Because their gene trees showed recent migration of haplotypes between islands and could not be relied upon to show the sequence of island colonization, each species was reduced to a single, multi-island taxon for DIVA analysis.

To test the effect of missing species on the biogeographical analysis, DIVA was also run on a tree consisting of the parsimony tree with the uncollected species inserted based on their morphological similarity to others. Two species, *H. mauiensis* and *H. nalo*, do not show sufficient characters to link them to others. *Hylaeus anomalus*, *H. finitimus*, *H. gliddenae*, *H. niloticus*, *H. perkinsianus* and *H. simplex* can be placed with relative certainty because their sister species are clear from morphology (Daly and Magnacca, 2003); *H. akoko*, *H. melanothrix*, *H. perspicuus* and *H. satelles* are somewhat less certain in their exact relationship to other species. For example, *H. akoko* shares features of both *H. fuscipennis* and *H. pubescens*, and could be placed as sister to either one, or to the two together. We have chosen the latter because *H. akoko* also has face marks, which are found in related members of the species group but not in *H. fuscipennis* and *H. pubescens*.

## Results

### *Heteroplasmy and cloning*

Cloning of polymorphic sequences produced some unusual results. All cloned sequences had bases that differed from unambiguous bases in the original, non-cloned sequence. These ranged in number from three to 20, too high to be explained by *Taq* error (although the original was unambiguous, the Maui representative of *H. unicus* was cloned because conspecific specimens were polymorphic; but its cloned sequence was identical to

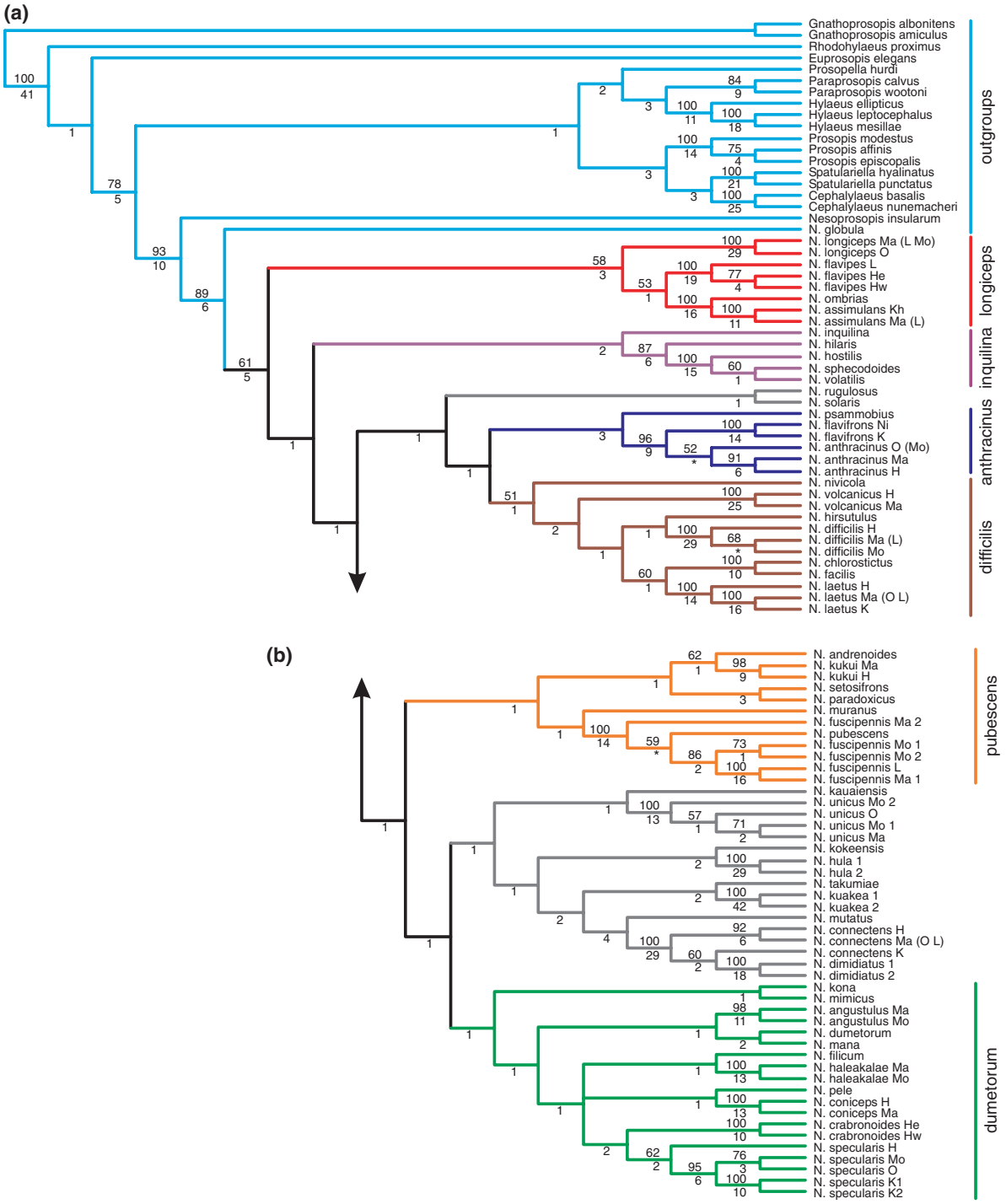


Fig. 2. Consensus of 16 most-parsimonious trees. Bootstrap values are above branches, Bremer support below; nodes marked with an asterisk for Bremer support had > 50% bootstrap support but collapse in the consensus tree. Colors indicate previously identified species groups; gray species were "unaffiliated" with a species group. Population identifiers: H = Hawaii (e = east, w = west), Ma = Maui, L = Lanai, Mo = Molokai, O = Oahu, K = Kauai, Ni = Niihau/Lehua. Island abbreviations in parentheses indicate those that were sequenced and found identical, and therefore not included as terminals.

the original). The anomalous differences were nearly all synonymous transitions, and no stop codons were evident. In specimens from which two or more cloned sequences were available (*H. dimidiatus*, *H. fuscipennis* from Maui and Molokai, *H. hula*, *H. kuakea*, *H. pubescens*, and *H. unicus* from Molokai), the

differences were sometimes shared. Furthermore, in a full analysis (not shown) all sequences came out next to the non-cloned sequence. A pseudogene origin for these aberrant sequences would require at least seven independent, very recent pseudogene origins within the small group. As this is highly unlikely and the sequences, like all others, have the characteristics of coding genes—no stop codons, insertions, deletions, or radical amino acid changes, and with a 2 : 1 : (5–10) ratio of first/second/third position changes relative to other sequences—we conclude that these sequences represent high levels of mitochondrial heteroplasmy within individuals. The anomalous differences imply that there are more than two mtDNA haplotypes in each individual, perhaps many more. This is supported by the fact that for the species with two clones available, they often did not cover both alternatives of clearly polymorphic bases in the original, non-cloned sequence.

Pseudogenes were present in the Japanese outgroups. Four species were originally attempted; two of the four (*H. insularum* and *H. matsumarai*) had pseudogenes that amplified to the exclusion of the true mitochondrial genes. A third species, *H. noomen*, did not amplify at all despite the near-universality of the primers among insects, suggesting possible interference from pseudogenes. The true (coding) mtDNA sequence for *H. insularum* was obtained by using "Rick" (Simon et al., 1994) as the forward primer. This resulted in 271 bases missing at the beginning of the sequence, the only missing data in the DNA data set. A clearly identifiable pseudogene sequence was also obtained from *H. kuakea* by cloning.

### *Genetic variation*

Base frequency was extremely A/T biased (Table 2), as is typical for insect mtDNA (Simon et al., 1994). Third positions averaged over 90% A/T; some outgroup species had no guanine at all in third positions. Third positions also show the largest differences between the minimum and maximum frequency for all bases.

Sequence divergence was high among the Hawaiian species. First position uncorrected distance between species varied from 0.5% to 12.5%; second, from 0% to 6.1%; third, from 7.4% to 30.9%; overall, from 3.0% to 15.0%. Translated amino acid variation ranged from 0.5% to 16.3%, an unusually high figure for a relatively young group.

Table 2
Base frequency (all sequences)

| | A | C | G | T |
|---|---|---|---|---|
| First | 36.94% | 11.99% | 18.42% | 32.66% |
| Second | 24.08% | 20.00% | 11.42% | 44.50% |
| Third | 40.99% | 4.62% | 3.35% | 51.03% |
| Overall | 34.01% | 12.20% | 11.08% | 42.71% |

There was usually considerable sequence variation between island populations of a single species. The sequences of all specimens from Lanai were nearly identical to those of the same species from Maui, but the islands of Hawaii, Maui, Molokai, Oahu and Kauai generally formed distinctly separate genetic entities. Exceptions (near-identical sequences from different islands) were *H. laetus* and *H. connectens* from Maui and Oahu (Molokai populations not collected for either species, but presumably also fitting here); *H. longiceps* from Molokai and Maui; and *H. anthracinus* from Molokai and Oahu (it is noteworthy that Molokai specimens of the latter two species were collected from the same site at the same time). Each of these pairs had nearly identical sequences (less than three bases difference). On the other hand, *H. assimulans* from Maui and Kahoolawe, which might be expected to be identical due to the close proximity of the islands, differed by 1.8% overall, and 4.8% at third positions.

Although large-scale sampling of individuals within a species was not done (except for Hawaii populations of the morphologically variable *H. coniceps* and *H. difficilis*, for which 10 and 17 individuals, respectively, were at least partially sequenced for identification), between two and seven individuals were sequenced for about half of the island populations. In nearly all cases there was no more than two or three bases separating pairs of individuals. However, disjunct populations of *H. crabronoides* and *H. flavipes* from the east and west sides of Hawaii had distinct sequences. Other species collected on both sides of the island and sequenced for multiple individuals (*H. coniceps*, *H. connectens*, *H. difficilis*, *H. dumetorum*, *H. pubescens* and *H. sphecodoides*) did not show such differences. Two individuals of *H. specularis* from Kauai collected from nearby sites, but on consecutive years, were substantially different (1.3% overall, 3.5% third positions). All distinct sequences were included in the analysis.

Despite the extensive intraspecific variation at the individual and species levels, the only two instances of shared mitochondrial haplotypes among island populations are within *H. unicus*, and among the closely related *H. akoko*, *H. fuscipennis* and *H. pubescens*. Both cases involve heteroplasmic species. In the former, two haplotypes from a single Molokai individual do not form a monophyletic unit. In the latter, all three species have polymorphisms at the same positions in the sequence, and one of the cloned sequences from *H. fuscipennis* comes out close to one from *H. pubescens* (*H. akoko* could not be cloned but likely has similarly mixed haplotypes). Mixing such as occurs in these two instances could be due to introgressive hybridization, incomplete lineage sorting, or both (Funk and Omland, 2003).

*Cladistic analysis*

Parsimony produced 16 shortest trees of length 4775. The strict consensus tree was highly resolved, though bootstrap and Bremer support was weak (Fig. 2). In addition, internal branches were extremely short relative to terminal branch lengths (Fig. 3). The arrangement of species into subgroups largely corresponds to previous morphological groupings (see Discussion).

*Biogeography*

DIVA analysis of all trees produced identical results (shown in Fig. 4). Because many nodes had multiple

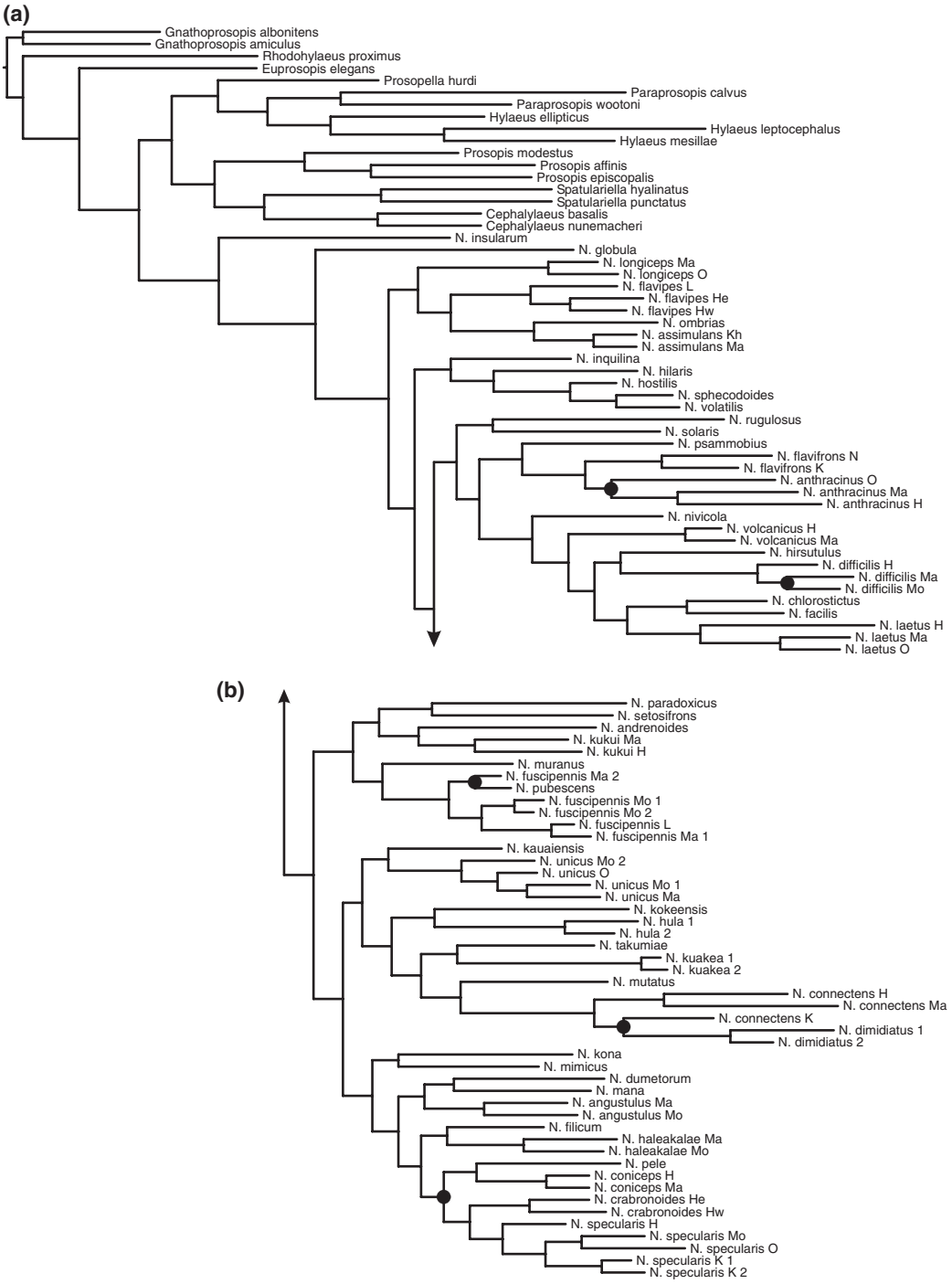


Fig. 3. One of the most-parsimonious trees showing relative branch lengths. Nodes that collapse in the consensus are indicated by black circles.

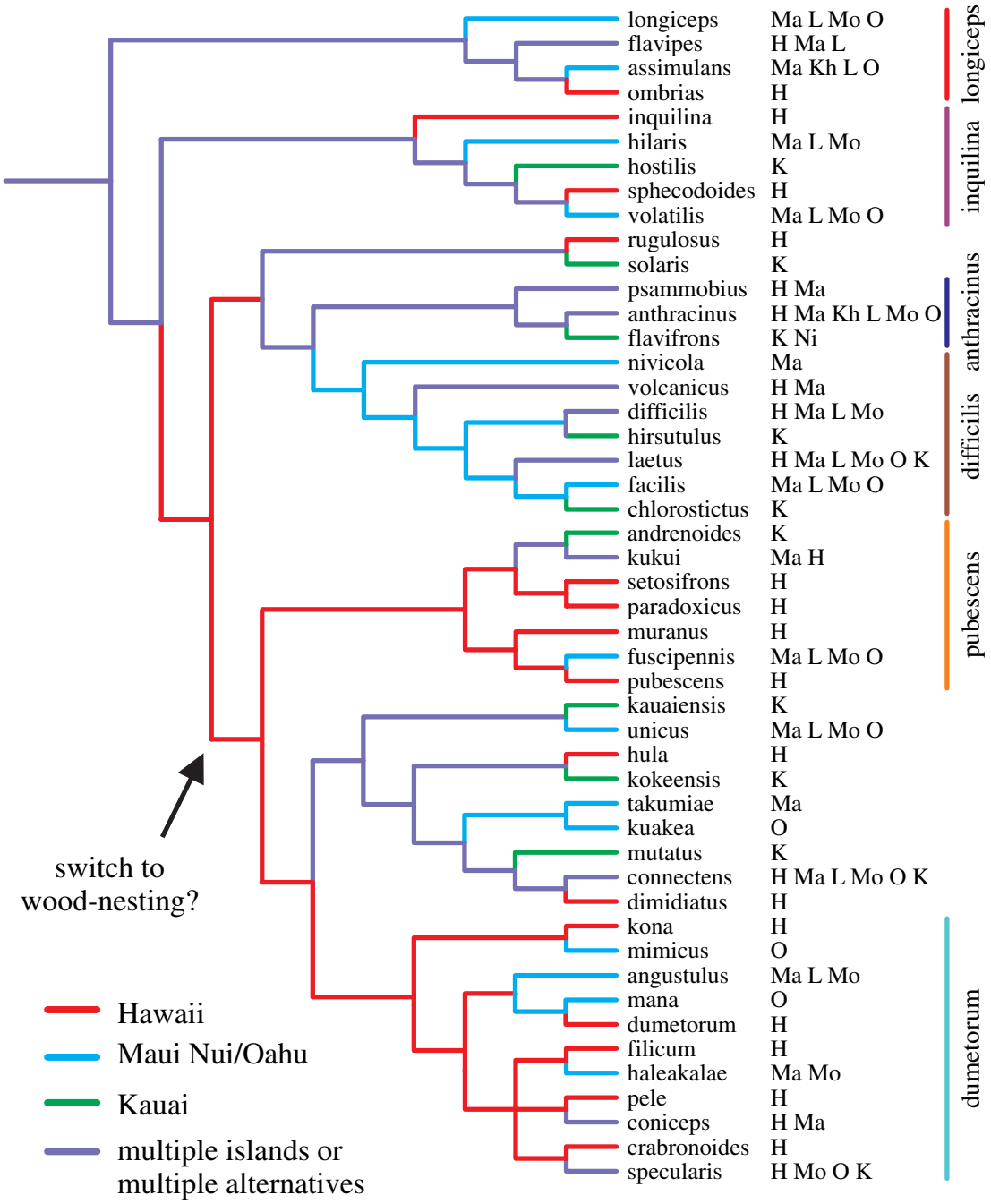


Fig. 4. Results of DIVA analysis of the parsimony trees. All terminals of one species are collapsed into a single branch. Terminal branches are the current distribution, indicated to the right. H = Hawaii, Ma = Maui, L = Lanai, Kh = Kahoolawe, Mo = Molokai, O = Oahu, K = Kauai, Ni = Niihau.

alternatives, the results are given only for those nodes where one or two alternatives were present. For the purpose of presentation, Maui Nui and Oahu are combined as a single color as few species are endemic to each (Fig. 1), and many (eight of 31) occur on all four islands.

The tree supports the hypothesis that nearly all of the early diversification, at least after separation of the two basal groups (the *longipes* and *inquilina* species groups), occurred on the island of Hawaii. Support for this hypothesis is particularly strong for the *dumetorum* and *pubescens* species groups, both of which are largely

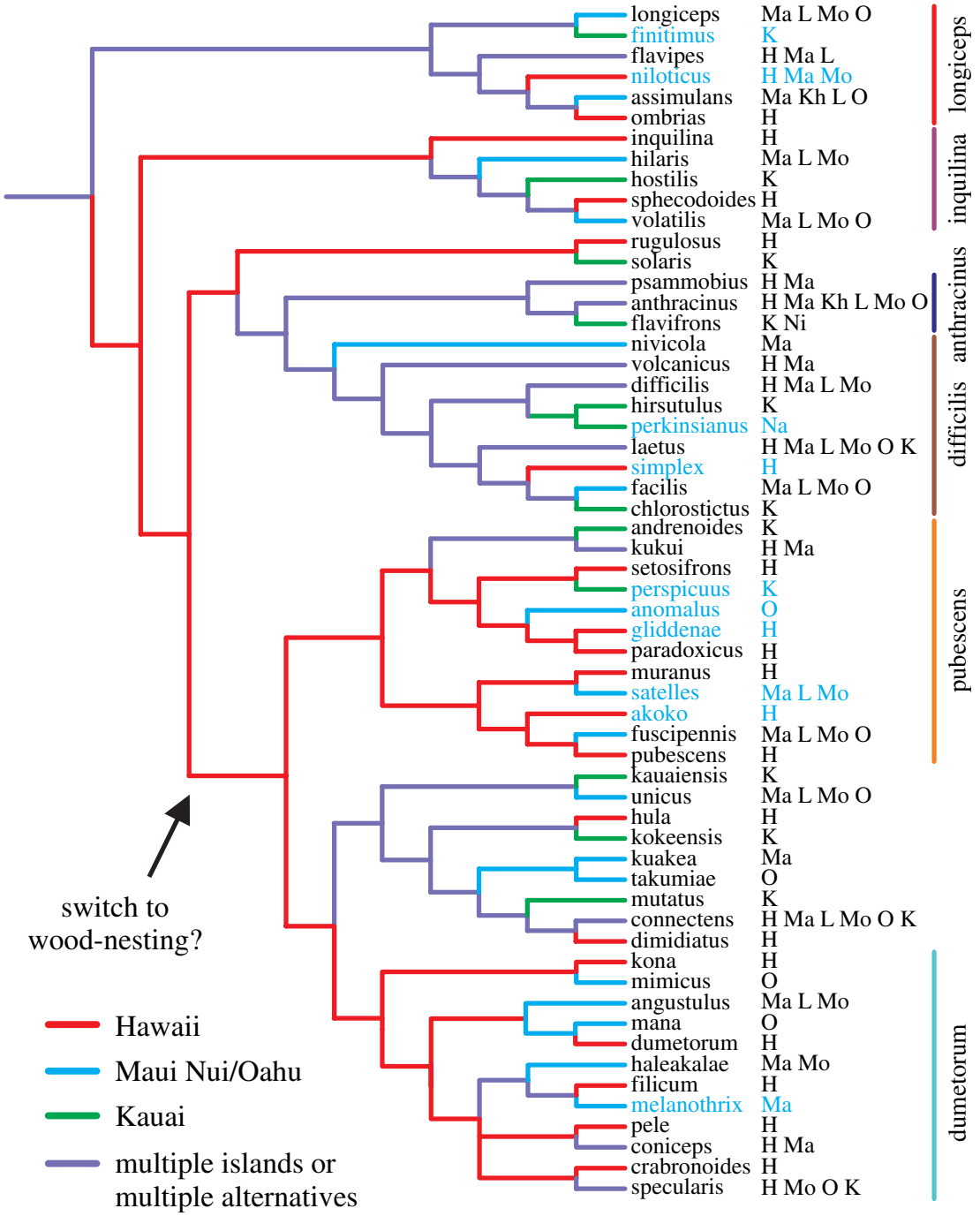


Fig. 5. Results of DIVA analysis on trees including taxa missing from phylogenetic analysis (shown in blue). See Fig. 4 caption for details of labeling. Na = Nihoa.

confined to wet forest. Diversification within other groups, especially those in the basal half of the tree, is more ambiguous. Inclusion of the missing species in the tree changed little in the biogeographical results (Fig. 5). It did, however, push back the unambiguous start of radiation on Hawaii island to include the *inquilina* group.

## Discussion

### *Genetic evolution*

The presence of a high rate of heteroplasmy (10 of 49 species, 20%, including *H. akoko*) was surprising. Previous studies focusing on heteroplasmy have usually

dealt with length polymorphism in the control region (Rand and Harrison, 1986; Kann et al., 1998). Heteroplasmy in coding genes has been found in other insects (Walton and Butlin, 1997), but has not been broadly investigated. The degree of differentiation was surprisingly high, with p-distances of 3–4% between haplotypes in a single individual. By comparison, most pairwise divergences of greater than 4% were between species, and most intraspecies comparisons were less than 4%.

Heteroplasmy, both in *Hylaeus* and in other taxa, may be underestimated due to chance and the quirks of PCR. It is well-known that even in a mixed or contaminated sample, if one particular sequence is replicated to an unusual degree at the beginning, it may end up dominating the final sample to such a degree that it appears to be the only one present. In several instances where sequencing had to be repeated, differing numbers of polymorphic sites showed up on the chromatograms. Similarly, in assembling gene contigs, one sequence would often be unambiguous where the overlapping fragment was clearly polymorphic at the same base. This kind of biased amplification could also explain the anomalous differences between the cloned sequences and apparently unambiguous bases in the uncloned sequences.

The existence of heteroplasmy may allow multiple haplotypes to persist (at least temporarily) and continue to diverge in a genetic environment where, if all individuals were homoplasmic, all but one would quickly be eliminated by chance. It is remarkable that despite the high degree of differentiation between haplotypes of heteroplasmic species, there was very little variation within island populations of species without heteroplasmy. The example of *H. specularis* on Kauai could be the exception that proves the rule: the relatively high degree of divergence between individuals caught at nearly the same site in consecutive years may be a brief phenomenon, where chance inheritance of haplotypes has resulted in homoplasmic individuals, but genetic sorting in the population has not yet run its course. Elucidation of the true cause and extent of heteroplasmy and other intra-island variation will require large-scale, population-level studies.

The high rate of amino acid change was the most striking genetic result. Amino acid differences of well over 10% were recorded between Hawaiian species (Table 3). Such divergence is equal to or greater than that found by Danforth (1999) across the halictid bee genus *Lasioglossum*, which includes over 1200 species worldwide (Michener, 2000). For example, the amino acid divergence between the North American *Lasioglossum (Lasioglossum) titusi* and Australian *L. (Chilalictus) erythrurum* is 4.49% over the 268 amino acids in common with this data set (the 3′ section of COI); even between *L. titusi* and *Halictus ligatus* it is only 8.24%. Over this same stretch in the Hawaiian *Hylaeus*, variation ranges up to 11.24%, with an average interspecies divergence of 5.12%. The greatest difference among the Hawaiian species for the entire data set (16.31%, between *H. connectens* Maui and *H. hirsutulus*), is almost as high as that between *Drosophila melanogaster* and *Anopheles gambiae* for the same stretch of DNA (16.43%). The lack of phylogenetic structure in the changes, combined with frequent back-and-forth changes among two or three amino acids at a site, suggest that relaxed constraint rather than selective advantage is driving the high rate of change.

The extreme rate of change in amino acids is especially notable when estimates of rates are applied. Pairwise comparisons of amino acid divergence between sister species or populations on Maui and Hawaii range from 1.34% up to 6.43% (Table 4). The island of Hawaii first emerged about 0.7 Myr ago (Moore and Clague, 1992), giving a minimum rate of change equivalent to 1.9–9.1%

Table 4
Hawaii–Maui amino acid divergence

| Species | Divergence |
|---|---|
| *anthracinus* | 1.34% |
| *connectens* | 3.49% |
| *difficilis* | 1.34% |
| *filicum-haleakalae* | 2.15% |
| *laetus* | 6.43% |
| *coniceps* | 1.04% |
| *pele-coniceps* (Hawaii) | 5.35% |
| *pele-coniceps* (Maui) | 4.28% |

Table 3
Amino acid divergence of rapidly evolving species. Figures are derived from uncorrected divergence in translated amino acids between the three listed species and all others; bottom line is for pairwise comparisons not involving those three species. Intraspecies figure for *H. dimidiatus* is between two heteroplasmic haplotypes

| Species | Average | Maximum | Minimum (interspecies) | Minimum (intraspecies) |
|---|---|---|---|---|
| *H. connectens* | 11.14% | 16.31% | 8.60% | 2.69% |
| *H. dimidiatus* | 11.23% | 14.44% | 9.09% | 1.34% |
| *H. laetus* | 9.21% | 12.30% | 5.36% | 1.87% |
| All others | 6.40% | 11.23% | 0.53% | 0.00% |

per Myr. Given that the island was probably inhospitable for hundreds of thousands of years after first emerging (see discussion of biogeography below), and there is no way to tell when separation between the populations took place, this must be considered a very conservative estimate. Thus, even if the biogeographical hypothesis of a recent origin for the entire group is incorrect, it is clear that genetic change is occurring very rapidly among them. Moreover, the existence of a large degree of variation among Hawaii–Maui divergence (Table 4) suggests that the lower rates are the result of more recent colonization of the island, and the higher rates closer to the true speed of evolution (note, however, that two of these involve the unusually fast-evolving species *H. connectens* and *H. laetus*; see Fig. 3). Unfortunately, lack of hierarchical biogeographical structure (see below) prevents an overall estimation of evolutionary rates as in Fleischer et al. (1998).

The only genetically non-monophyletic species were *H. fuscipennis* and *H. pubescens*, and *H. connectens* and *H. dimidiatus* (Fig. 2). The former is probably the result of introgressive hybridization (Funk and Omland, 2003): most *H. fuscipennis* genotypes cluster together, but one is most similar to *H. pubescens*, lacking the synapomorphies that unite the other *H. fuscipennis* genotypes. Similar events caused a great deal of confusion in phylogenetic estimation of Hawaiian *Laupala* crickets (Shaw, 1996, 2002). In that case, horizontal transfer of mitochondrial DNA between sympatric species caused by widespread hybridization led to the appearance of species from one island being most closely related to one another, a conclusion that was later shown to be incorrect by analysis of nuclear DNA (Shaw, 2002). In *Hylaeus*, problems with hybridization are not significant; the case of *H. fuscipennis* and *H. pubescens* is the only apparent instance of horizontal transfer in the data set, and the long-terminal branch lengths in other species argue against any recent or hidden gene transfer.

The paraphyly of *H. connectens* haplotypes relative to those of *H. dimidiatus* appears to represent a case of recent speciation, despite their very different appearance and habits. Although *H. connectens* occurs in dry forest on Hawaii, it has not been found sympatric with *H. dimidiatus*. Despite their differences, the strong monophyly of the *H. dimidiatus* haplotypes and long branches leading to *H. connectens* populations make lineage sorting or introgression unlikely explanations for paraphyly. The long branch uniting all populations involved, and several unique amino acid changes along this branch, also makes long-branch attraction a very remote possibility. With the high degree of differentiation between *H. connectens* populations, *H. dimidiatus* may be the result of *H. connectens* from Kauai secondarily dispersing to Hawaii, being unable to breed with the *H. connectens* there, and taking a separate evolutionary path.

### *Phylogenetics*

The previously recognized informal species groups were largely confirmed by the tree (Fig. 2). The *longiceps*, *difficilis*, *inquilina*, *anthracinus*, *pubescens* and *dumetorum* groups can be approximately defined using facial markings, setation, and habitat, though their exact composition was not always clear. For example, *H. kokeensis* is most similar overall to *H. mana* and *H. mimicus* in the *dumetorum* group, but lacks the grooved scape that is characteristic of the group.

One of the most striking results of the tree was that nearly all the species that did not clearly fit into one of the above-mentioned species groups formed a single clade. This grouping (which includes *H. kokeensis*, as well as *H. connectens* and *H. unicus*) has little in common morphologically, and most of its members are poorly known; three of the nine species were only described in 2003. Owing to the lack of any synapomorphies, it is not considered a reliable species group. Still, it is interesting that nearly all the unassociated species form a single clade, especially when one considers that the group is dominated by, and includes most of, the species that seem to favor mesic areas.

Other parts of the tree do show that rapid morphological change is possible, especially if one considers the possibility of extinct intermediates. For example, *H. paradoxicus* and *H. setosifrons* appear very different; yet with the inclusion of the now-rare or extinct *H. anomalus* and *H. gliddenae*, an obvious transformation series is apparent in body color and male facial markings (Daly and Magnacca, 2003). The strongly supported position of *H. dimidiatus* as an offshoot of *H. connectens* also indicates the potential for different-looking species to be closely related. *Hylaeus connectens* occurs in all habitats on all islands (though preferring montane mesic areas), is relatively robust, usually lacks yellow marks on the body, and has facial marks only on the clypeus and sometimes narrowly adjacent (similar to *H. difficilis*); *H. dimidiatus* is restricted to small areas of montane dry forest on Hawaii, is smaller and much more gracile, and has conspicuous yellow markings on the pronotum and facial marks filling the lower face and extending a bit up the eye (similar to *H. kokeensis*).

### *Biogeography*

The most distinctive characteristic of the tree was the almost complete lack of any pattern in terms of species distribution. In most Hawaiian clades of both plants and insects, the most basal branches consist of species found on the oldest islands of Kauai and Oahu (Wagner and Funk, 1995; Liebherr and Zimmerman, 2000). This arrangement indicates arrival by a founder at least 3–5 Myr ago, followed by speciation by "island-hopping", as populations (which may later become distinct species)

are established on new islands as they arise. If the first colonist arrived more recently, when most or all islands were subaerial, then migration between islands—and subsequent speciation—would have been more or less random (Lowrey, 1995). In *Hylaeus*, it is the latter scenario that is supported by the tree: there is no clear pattern of island-to-island dispersal.

Despite the lack of a hierarchical structure, the main mode of recent speciation still appears to be through dispersal and isolation of new island populations. Recent speciation within an island, as occurs in more sedentary taxa such as ground beetles (Liebherr and Zimmerman, 2000) is rare. The only unambiguous example (i.e., where a pair of sister species are both restricted to a single island) is *H. gliddenae* and *H. paradoxicus* (Daly and Magnacca, 2003). On the other hand, there are several pairs or trios of species that are "complementary" in the islands they inhabit (Table 5).

However, when one looks at the history of diversification in the group, it is apparent that considerable intra-island speciation occurred on Hawaii island following divergence of the *longiceps* group (and possibly the *inquilina* group). This includes separation of the progenitor population into four major lineages—the *difficilis–anthracinus–rugulosus*, *pubescens*, *kauaiensis–connectens* (possibly due to dispersal to Kauai), and *dumetorum* clades—and early diversification in the *pubescens* and *dumetorum* groups (Figs 3 and 4). Such intra-island speciation is consistent with rapid diversification into ecologically distinct species groups as the first step of adaptive radiation, a notion supported by the short basal branch lengths (Fig. 3).

The strong support for early radiation of this group on the island of Hawaii is a surprising result, as it is the youngest island of the archipelago (Clague, 1996). Such a recent origin requires an exceedingly high rate of genetic evolution to attain the observed degree of divergence. However, the differences observed between Hawaii–Maui island populations and sister species (Table 4) demonstrates unequivocally that unusually rapid genetic change is taking place. Thus, speed of genetic evolution is not a factor in evaluating the plausibility of biogeographical conclusions.

Nevertheless, evolution from a single individual into 60 species in such a short time still requires relatively rapid speciation. Based on the tree topology, a minimum of 11 speciation events are required to reach *H. chlorostictus* (the longest series of nodes in the tree). If the first colonist arrived about 0.5 Myr ago, it would require a speciation event every 45 000 years (with no extinctions, at least along this line) to reach the number of species known today. While such a rate may seem fast on a geological time-scale, it is easily possible, especially considering that several of the earliest splits would have occurred very quickly as part of the first radiation into a new habitat. Indeed, such rapid evolution has been demonstrated in Hawaii: five species of *Omiodes* moths are specific to banana (Zimmerman, 1958, 1960), a plant that only arrived with the Polynesians no more than 1700 years ago (Kirch, 1985). In addition, there are dozens, if not hundreds, of island-specific species on Molokai, Lanai and Maui, islands that were joined as recently as 20 000 years ago (Price and Elliott-Fisk, 2004).

The first volcano of the island of Hawaii to break the surface, about 0.7 Myr ago, is known as Mahukona (Moore and Clague, 1992). It is now submerged, and even at its peak about 0.5 Myr ago, Mahukona was only about 250 m high (Clague, 1996). At that time it was probably largely covered with recent lava flows, supporting only low-diversity dry forest, and it subsided beneath the surface by 0.4 Myr ago (Clague, 1996). It was not until 0.5–0.4 Myr ago that Kohala volcano would have reached sufficient height to generate rainfall, allowing mesic and wet forest to develop on the island (Carson and Clague, 1995). The biogeographical hypothesis supported by the tree requires that the switch to wood-nesting and wet forest inhabitation in the *pubescens* and *dumetorum* groups must have taken place after this time (Figs 4 and 5). If the very first immigrant had arrived prior to the development of rainforest on Hawaii, adaptation to that habitat would certainly have taken place on another island. Therefore, the inescapable conclusion is that the first arrival of *Hylaeus* in Hawaii occurred less than 500 000 years ago.

Thus, the sum of the evidence supports the following scenario. The first *Hylaeus* arrived on the island of Hawaii somewhere about 0.5–0.4 Myr ago, or possibly even later. The island would have to have been at least 500–1000 m high at the time in order to possess all the necessary ecosystems. Even before reaching other

Table 5
Trios and pairs of species with "complementary" distributions on various islands. Each row represents a group of closely related species (see Fig. 5 for relationships)

| Species group | Kauai | Oahu + Maui Nui | Hawaii |
|---|---|---|---|
| *inquilina* | *H. hostilis* | *H. volatilis* | *H. sphecodoides* |
| *difficilis* | *H. chlorostictus* | *H. facilis* | *H. simplex* |
| *anthracinus* | *H. flavifrons* | *H. anthracinus* | *H. anthracinus* |
| | *H. kauaiensis* | *H. unicus* | |
| *longiceps* | *H. finitimus* | *H. longiceps* | |
| *longiceps* | | *H. assimulans* | *H. ombrias* |

islands (or after only the ancestor of the *longiceps* group split off), the descendants of the initial (presumably ground-nesting) colonist separated into ecological specialists. These included coastal strand and dry forest (*longiceps* and *anthracinus* groups); mid-elevation and montane dry shrubland (*difficilis* group); cleptoparasites (*inquilina* group); and wood nesters, which primarily took to wet and mesic forest (Daly and Magnacca, 2003). Once these major groups had diverged, dispersal to other islands began. In the *pubescens* and *dumetorum* groups, further speciation took place on Hawaii, probably as a result of these more adaptable wood-nesting species separating into specific ecological zones, from extremely wet to mesic and even dry forest. Eventually, relative stasis set in, and some species began expanding their ranges beyond the island they originated on, but not (as yet) separating into distinct species.

There is no question that this hypothesis would sit better with stronger support for the tree. Nuclear genes have often been found to be better than mitochondrial at resolving phylogenies (Brower and DeSalle, 1998; Baker et al., 2001), but four genes have been tried and found to lack sufficient variation (unpubl. data). Although other mitochondrial genes have been shown to perform better than COI and COII (Corneli and Ward, 2000; Shevchuk and Allard, 2001), an exceedingly high rate of change was the primary problem here, and one that would be exacerbated in the faster-evolving ATPase and NADH dehydrogenase genes. A segment of ND4 was sequenced for several species and found to be evolving at approximately 1.5 times the rate of the sequence used in this study (data not shown). Morphological characters are probably more useful, but discrete, consistent characters are few and far between.

However, evidence independent of the phylogenetic hypothesis supports the idea of a recent, Hawaii-centered origin for *Hylaeus*. The very fact that strikingly little variation was found in all of the nuclear genes, even in introns (unpubl. data), is indicative of a very recent origin. The extremely short internal and long terminal branch lengths support this as well, suggesting a very rapid radiation into at least half as many species as exist today, followed by relative stability of species and dispersal-related speciation (though this could also be a result of rapid change obscuring older synapomorphies). The *dumetorum* and *pubescens* groups, both of which are well-supported by morphological characters, exhibit a general pattern in pairs of sister species, where one occurs on Hawaii and the other on another island, or on both Hawaii and other islands. With such a distribution, the relationship between those species pairs hardly matters: the most parsimonious conclusion involves a first round of speciation on Hawaii island, followed by dispersal that led to the establishment of sister species on other islands. The confirmation that both of these groups are rooted on Hawaii thus supports placing the development of wood-nesting on that island as well. Therefore, it can be concluded with confidence that at a minimum, the clade consisting of the 34 wood-nesting species originated and radiated on Hawaii; and with the genetic imprint of a rapid radiation as discussed above, it is almost certain that the entire group had its origin there.

One of the most striking implications of *Hylaeus* radiation on the island of Hawaii is the rate of speciation. A recent study of the Hawaiian cricket genus *Laupala* (Mendelson and Shaw, 2005) found a clade of six Hawaii island species; using the formula $r = \ln N/t$ (where $r$ = speciation rate, $N$ = number of species in the clade, and $t$ = date of the most recent common ancestor; McCune, 1997), they calculate the speciation rate of this group as 4.17 species per Myr. It should be noted that they use 0.43 Myr as the age of the island; this is the age of the oldest exposed rocks, but the island is much older (Moore and Clague, 1992; but see Discussion above on habitat suitability). Using this same formula and date for comparison, the minimum speciation rate for the hypothesis in Fig. 5 (that is, the entire radiation except the *longiceps* group taking place on Hawaii) is 9.23 species per Myr. This is over twice the rate for *Laupala* (Mendelson and Shaw, 2005), which is claimed to be the highest rate ever found except for cichlid fish (McCune, 1997). Even looking at smaller clades but assuming the same age (Table 6), the speci-

Table 6
Comparison of speciation rates on the island of Hawaii for different clades. Rate is species per million years (see text for formula), calculated using 0.43 Myr as the age of the island. Rates are minimums; arrival of the clade ancestor on the island after this date would mean a higher rate. Analysis of the tree with sequenced species only supports a Hawaii origin for the *anthracinus/difficilis* through *dumetorum* clade, while inclusion of missing species supports it for the *inquilina* through *dumetorum* clade (see Fig. 1)

| | Sequenced species only | | Including missing species | |
|---|---|---|---|---|
| Species groups | *n* | rate | *n* | rate |
| *inquilina* through *dumetorum* | 44 | 8.80 | 53 | 9.23 |
| *anthracinus/difficilis* through *dumetorum* | 39 | 8.52 | 48 | 9.00 |
| *pubescens* through *dumetorum* (wood nesters) | 28 | 7.75 | 34 | 8.20 |
| *pubescens* | 7 | 4.53 | 12 | 5.78 |
| *dumetorum* | 12 | 5.78 | 13 | 5.96 |

ation rate is still higher than that of the *Laupala* clade. Considering the high likelihood that some species have gone extinct or remain undiscovered, these rates are certainly conservative.

Sexual selection based on male songs is used to explain the high rate of speciation among the crickets (Mendelson and Shaw, 2005). There is no evidence for any kind of unusually intense sexual or other non-adaptive selection among *Hylaeus*. It is more likely that rapid expansion into open niches in a novel habitat is sufficient to explain the rapid diversification of *Hylaeus*. Such a scenario is also more consistent with the short basal and long-terminal branches of the trees. The *Hylaeus* may therefore be a more typical illustration of what happened in the early evolution of many insect groups in Hawaii and other remote islands.

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## Appendix 1: morphological characters

1. Male paraocular mark. State 1 found in the *difficilis* and *anthracinus* groups, and in *H. connectens* and *H. kuakea*; states 2 and 3 found in several species independently.
   0 highest adjacent to eye
   1 highest adjacent to clypeus
   2 absent
   3 evenly filling in paraocular area
2. Male frons setation. State 1 is characteristic of the *dumetorum* group.
   0 even
   1 with a triangular or rhomboid patch of dense black setae
3. Underside of male scape. State 1 is characteristic of the *dumetorum* group, sometimes developed into state 2. State 3 occurs in the *pubescens* group. All of the modifications are probably associated with glands in the scape.
   0 smooth, flat
   1 with a distinct groove near the medial edge
   2 with a broad depression or open groove with glandular ducts
   3 with an open, round pit
4. Male scape setation. State 1 is found in the *flavipes* and *difficilis* groups, with the exception of *H. laetus*.
   0 glabrous
   1 underside covered with short, erect setae
5. Female paraocular mark. State 2 found in most members of the *dumetorum* group and a few other species; state 1 may be intermediate, but the three states seem distinct.
   0 absent
   1 variable within the species: sometimes a small spot present (never large), sometimes absent
   2 a relatively broad stripe always present
6. Female labial fovea. State 1 is found in all members of the *dumetorum* and *pubescens* groups, and a few others. Apparently correlated with wet forest habit.
   0 narrow, edges fading before the base
   1 broad, edges distinct to the base
7. Female mandibular teeth. State 1 found in most members of the *pubescens* group, and in *H. anthracinus* and *H. flavifrons*. State 2 found only in *H. rugulosus*.
   0 two teeth
   1 three teeth
   2 no teeth
8. Female protarsal setae. These setae are used for collecting pollen; state 1 is characteristic of the parasitic clade (*inquilina* group).
   0 long, erect, curved at tip
   1 short, prostrate, and straight
9. Procoxal lamella. State 1 is found in the three derived parasitic species. It is more prominent in the female.
   0 lamella narrow or not apparent
   1 lamella broad, with a basal supporting spine
10. Propodeal sculpture. State 1 found mainly in the *dumetorum* group.
   0 lineate or reticulate
   1 mostly smooth coriaceous
11. T2 gradulus. Distribution similar to female labial fovea.
   0 distinct
   1 indistinct or absent
12. T3 punctation. State 1 is characteristic of the *pubescens* group. Also found in *H. haleakalae* and *H. kauaiensis*.
   0 impunctate
   1 distinctly punctate
13. T6 setae. State 0 found in the *difficilis*, *flavipes*, and *inquilina* groups; state 1 in nearly all others. A few have the intermediate state 2; state 3 occurs only in *H. pubescens*.
   0 pale to brown, densely plumose, prostrate and relatively short
   1 black, sparsely plumose, erect
   2 black, sparsely plumose, prostrate
   3 red-brown, very dense, plumose, prostrate
14. Male S8. State 1 is characteristic of the *difficilis* group. State 2 is restricted to the outgroups.
   0 apical process dorsoventrally dilated, usually humped
   1 apical process very narrow, rod-like, evenly and highly arched
   2 apical process flat, no dorsoventral membrane.

## Appendix 2

Morphological character matrix

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Euprosopis elegans* | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Gnathoprosopis albonitens* | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Gnathoprosopis amiculus* | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Rhodohylaeus* sp. | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | – |
| *Cephalylaeus basalis* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | – |
| *Cephalylaeus nunemacheri* | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | – |
| *Hylaeus leptocephalus* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| *Hylaeus ellipticus* | 0 | 0 | 0 | 0 | 2 | 0 | – | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| *Hylaeus mesillae* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Paraprosopis calvus* | 0 | – | – | – | 2 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| *Paraprosopis wootoni* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| *Prosopella hurdi* | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| *Prosopis affinis* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 |
| *Prosopis episcopalis* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Prosopis modestus* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Spatulariella hyalinatus* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| *Spatulariella punctatus* | 1 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| *Nesoprosopis globula* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | – | 0 | 0 | 0 |
| *Nesoprosopis insularum* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| *Nesoprosopis andrenoides* | 2 | 0 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| *Nesoprosopis angustulus* | 0 | 1 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis anthracinus* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Nesoprosopis assimulans* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis chlorostictus* | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis coniceps* | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis connectens* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| *Nesoprosopis crabronoides* | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis difficilis* | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis dimidiatus* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| *Nesoprosopis dumetorum* | 0 | 1 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis facilis* | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis filicum* | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis flavifrons* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Nesoprosopis flavipes* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis fuscipennis* | 2 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| *Nesoprosopis haleakalae* | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| *Nesoprosopis hilaris* | 0 | 0 | 0 | 0 | 0 | – | – | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis hirsutulus* | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis hostilis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis hula* | 3 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| *Nesoprosopis inquilina* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis kauaiensis* | 3 | 0 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| *Nesoprosopis kokeensis* | 0 | 0 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis kona* | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis kuakea* | 1 | 0 | 0 | 0 | – | – | – | 0 | 0 | 0 | 0 | 0 | – | 0 |
| *Nesoprosopis kukui* | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Nesoprosopis laetus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis longiceps* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis mana* | 0 | 1 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis mimicus* | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis muranus* | 2 | 0 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| *Nesoprosopis mutatus* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| *Nesoprosopis nivicola* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis ombrias* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis paradoxicus* | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | – | 1 | 1 | 0 |
| *Nesoprosopis pele* | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis psammobius* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Nesoprosopis pubescens* | 2 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| *Nesoprosopis rugulosus* | 2 | 0 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis setosifrons* | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| *Nesoprosopis solaris* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

## Appendix 2

*Continued*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Nesoprosopis specularis* | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis sphecodoides* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Nesoprosopis takumiae* | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Nesoprosopis unicus* | 3 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Nesoprosopis volcanicus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Nesoprosopis volatilis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |