



Low nuclear DNA variation supports a recent origin of Hawaiian *Hylaeus* bees (Hymenoptera: Colletidae)

Karl N. Magnacca^{*,1}, Bryan N. Danforth

Department of Entomology, Cornell University, Ithaca, NY 14853, USA

Received 22 May 2006; revised 14 August 2006; accepted 4 September 2006

Abstract

Previous phylogenetic work on the Hawaiian bees of the genus *Hylaeus*, based on mitochondrial DNA and morphology, appeared to support a recent origin for the group, but support for the resulting tree was weak. Four nuclear genes with varying evolutionary rates—arginine kinase, EF-1 α , opsin, and *wingless*—were sequenced for a reduced taxon set in an attempt to find one or more data set that would provide better support. All showed very low variation (<2%) in the ingroup. Comparison among genes revealed a much higher than expected rate of evolution in mtDNA, especially at first and second positions. While the data from the nuclear genes showed insufficient variation for phylogenetic analysis, the strong sequence similarity among the Hawaiian species supports the previous hypothesis of a recent origin for the group.

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Keywords: *Hylaeus*; Colletidae; Nuclear DNA; Speciation; Hawaii; Biogeography

1. Introduction

Mitochondrial genes have long been used for molecular phylogenetics, due to their relatively rapid rate of evolution, general lack of polymorphism, and the availability of universal primers (Simon et al., 1994). However, their use entails several disadvantages, notably a tendency to have strong A–T bias that leads to a high degree of homoplasy and saturation at low levels of divergence (Brower and DeSalle, 1994, 1998). In recent years the use of nuclear gene sequences for phylogeny reconstruction has blossomed as widely-usable primers are developed for more and more genes (Baker and DeSalle, 1997; Friedlander et al., 1994; Moulton and Wiegmann, 2004). The use of mitochondrial DNA (mtDNA) has decreased as a result, especially with the publication of data showing that many, if not all, mito-

chondrial genes are inferior to nuclear genes with regard to resolution and congruence (Baker et al., 2001; Corneli and Ward, 2000; Lin and Danforth, 2004; Shevchuk and Allard, 2001). Many phylogenies constructed with mitochondrial sequences have been reanalyzed with the addition of nuclear sequences, sometimes with highly incongruent results (Shaw, 1996, 2002).

The native bee fauna of Hawaii consists of 60 species of *Hylaeus* (*Nesoprosopis*) derived from a single colonist (Daly and Magnacca, 2003). Efforts to develop a phylogenetic hypothesis for the Hawaiian bees using mitochondrial DNA (Magnacca and Danforth, 2006) resulted in a weakly supported tree due to apparently rapid speciation, base composition bias, and a high rate of change with resulting homoplasy. Biogeographic analysis using DIVA (Ronquist, 1997) strongly suggested a recent origin for the group based on placement of species-group ancestors on the island of Hawaii. This would require that most, if not all, of the basal radiation of *Hylaeus* in the Hawaiian Islands occurred within the last 0.5–0.7 million years (Moore and Clague, 1992). Such a conclusion is surprising given the large number of species currently known, and the high degree of

* Corresponding author. Fax: +1 510 643 5438.

E-mail address: magnacca@nature.berkeley.edu (K.N. Magnacca).

¹ Present address: Department of Environmental Science, Policy, and Management, University of California-Berkeley, Berkeley, CA 94720-3114, USA.

divergence in mtDNA sequences. Moreover, it would be unusual in the Hawaiian biota, as many large insect and plant radiations are characterized by basal divergences associated with the older islands (Liebherr and Zimmerman, 1998; Wagner and Funk, 1995).

Building support for correct estimation of the time of origin and subsequent radiation of the group is important, and has broader significance for Hawaiian biogeography and evolution. Previous biogeographic studies of Hawaiian insects have involved groups that are relatively sedentary, such as the forest-dwelling *Drosophila* and flightless beetles and crickets (DeSalle, 1995; Liebherr and Zimmerman, 1998; Shaw, 2002), or of modest diversity, such as the *Megalagrion* damselflies (Jordan et al., 2003). *Hylaeus* is the first diverse group of highly vagile insects to be investigated biogeographically. Moreover, as the only representatives in Hawaii of a group that is crucial to the survival of many plants elsewhere, it is clear that the time of their arrival is an important factor in assessing the evolutionary history of plants in the islands.

In analyzing the mtDNA data for *Hylaeus*, it became clear that unusually rapid gene evolution was taking place (Magnacca and Danforth, 2006). Divergence between island populations of a single species was high, and heteroplasmy was present in about 20% of the species. In addition, high rates of homoplasious amino acid change indicated that the proteins encoded by the genes (COI and COII) had unusually low functional constraint. Therefore, we evaluated four prospective nuclear genes for their usefulness in building a more strongly supported phylogeny: elongation factor-1 α (EF-1 α); arginine kinase (ArgK); long-wavelength rhodopsin (opsin); and *wingless*. All have

been used for phylogenetic studies in various insect groups, including bees (Ascher et al., 2001; Brower and DeSalle, 1998; Danforth et al., 1999; Kawakita et al., 2003; Mardulyn and Cameron, 1999). It was hoped that a slower-evolving nuclear gene with lower base composition bias would provide better phylogenetic information, at least for relationships among species-groups.

2. Materials and methods

2.1. Taxon sampling

A subset of the complete species data set (Magnacca and Danforth, 2006) was used to test the usefulness of the four genes (Table 1). Species were selected to span the entire Hawaiian radiation and to include as much diversity as possible. One species was selected from each of the morphologically-based species groups (the *longiceps*, *inquilina*, *anthracinus*, *difficilis*, *pubescens*, and *dumetorum* groups of Magnacca and Danforth (2006)). Six species not clearly affiliated with a species group were also included, for a total of 12 ingroup taxa. Outgroups included one Japanese *Nesoprosopis*; two Holarctic *Hylaeus* subgenera, *Hylaeus s.s.* and *Spatulariella*; and two Australian *Hylaeus* subgenera, *Euprosopis* and *Gnathoprosopis*.

2.2. DNA extraction

Total DNA was extracted using standard protocols (Doyle and Doyle, 1990). Tissue was taken from the whole body, thoracic musculature or reproductive organs. Samples were macerated in individual 1.5 ml Eppendorf tubes

Table 1
Specimens used for sequencing

	State/Island	Locality	Date	Collector
Australian outgroups				
<i>H. (Gnathoprosopis) amicus</i>	S. Australia	10 km E Kimba	5 Jan 1999	B.N.D
<i>H. (Euprosopis) elegans</i>	S. Australia	10 km E Kimba	5 Jan 1999	B.N.D
Holarctic outgroups				
<i>H. (Hylaeus) leptcephalus</i>	New York	Cornell University	27 Jul 1999	J. Ascher
<i>H. (Spatulariella) punctatus</i>	California	U.C. Berkeley campus	21 Jun 1999	J. Ascher
Japanese <i>Nesoprosopis</i>				
<i>H. (Nesoprosopis) globula</i>	Japan	Inohara-kogen Yokota-cho	10 Oct 1999	Y. Maeta
Hawaiian <i>Nesoprosopis</i>				
<i>H. (Nesoprosopis) anthracinus</i>	Hawaii	South Point	18 Jul 1999	KNM
<i>H. (Nesoprosopis) connectens</i>	Kauai	Polihale State Park	25 Aug 1999	KNM
<i>H. (Nesoprosopis) difficilis</i>	Hawaii	HAVO, Mauna Loa Rd.	2 Jan 1999	KNM
<i>H. (Nesoprosopis) hula</i>	Hawaii	HAVO, Tree Molds	10 Aug 1999	KNM
<i>H. (Nesoprosopis) inquilina</i>	Hawaii	HAVO, 0.9 mi. S Mauna Loa Rd.	4 Jan 1999	KNM
<i>H. (Nesoprosopis) kauaiensis</i>	Kauai	Alakai Swamp Trail	3 Jul 1999	KNM
<i>H. (Nesoprosopis) kokeensis</i>	Kauai	Kokee Rd.	24 Aug 1999	KNM
<i>H. (Nesoprosopis) kona</i>	Hawaii	Kipuka Alala	14 Jul 1999	KNM
<i>H. (Nesoprosopis) longiceps</i>	Oahu	Kaena Point NAR	12 Jun 1999	KNM
<i>H. (Nesoprosopis) pele</i>	Hawaii	Kipuka Alala	14 Jul 1999	KNM
<i>H. (Nesoprosopis) pubescens</i>	Hawaii	HAVO, Devastation Trail	8 Jan 1999	KNM
<i>H. (Nesoprosopis) solaris</i>	Kauai	Polihale State Park	25 Aug 1999	KNM

HAVO, Hawaii Volcanoes National Park; NAR, Natural Area Reserve (State of Hawaii).

Table 2
Nuclear gene primer pairs (5' end to left)

Primer name	Direction	Sequence
ArgK for2	Forward	GACAGCAARTCTCTGCTGAAGAA
ArgK rev2	Reverse	GGTYTTGGCATCGTTGTGGTAGATAC
EF1 For1deg	Forward	GYATCGACAARCGTACSATYG
EF1 F2rev1	Reverse	AATCAGCAGCACCTTTAGGTGG
EF1 For3	Forward	GGNGACAAYGTTGGYTTCAACG
EF1 Cho10mod	Reverse	ACRGCVACKGTYTGHCKCATGTC
OpsinF	Forward	AATTGCTATTAYGARACNTGGGT
OpsinR	Reverse	ATATGGAGTCCANGCCATRAACCA
Wg1a	Forward	GARTGYAARTGYCAYGGYATGTCTGG
Wg2a	Reverse	ACTICGCARCACCARTGGAATGTRCA

with 2× CTAB extraction buffer and 100 µg proteinase K. Samples were incubated for 2 h at 55 °C, extracted with 24:1 chloroform–isoamyl alcohol, digested for 30 min. with 10 µg RNase, and extracted again with 25:24:1 phenol–chloroform–isoamyl alcohol and chloroform–isoamyl alcohol. DNA was precipitated with 2.5 volumes of ice-cold 100% ethanol and 0.1 volume of 3 M sodium acetate, washed with 80% ethanol, and resuspended in 50 µl Tris–EDTA (pH 7.6) buffer.

2.3. PCR and sequencing

Mitochondrial sequences of COI, tRNA-Leu, and COII, and morphological characters, are those from Magnacca and Danforth (2006). These include 822 bp of the 3' end of COI, tRNA-Leu, and 300 bp of the 5' end of COII. Cloned sequences were used for *Hylaeus hula* (clone 2 used), *Hylaeus kokeensis*, and *Hylaeus pubescens*, which had large num-

bers of heteroplasmic polymorphisms in the mtDNA. Nuclear genes were sequenced using the primers listed in Table 2. PCR products were gel-purified, then purified for sequencing with the Promega (Madison, WI) Wizard PCR Preps DNA Purification Kit. 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. Each segment was sequenced in both directions except for *wingless*, which was only 450 bp and only sequenced in the forward direction. GenBank Accession Numbers for nuclear sequences are provided in Table 3.

2.4. Analysis

Sequences were aligned with the Clustal V algorithm in MegAlign 5.06 (DNASstar) and checked by eye. Maximum parsimony (MP) analyses were carried out using PAUP* 4.0b10. MP searches were conducted with 100 random addition sequence replicates, holding a maximum of 30 trees at each step, followed by searching with the option “search all trees in memory”. Gaps were treated as missing data; none gave any indication of being shared among taxa, except for a 6 bp deletion in EF-1α that appears to be a synapomorphy for *Nesoprosopis*. Winclada 1.00.08 was used to create branch length trees. Parsimony analyses were run for: (1) each nuclear gene separately; (2) the mitochondrial data set; (3) the morphological data set; (4) all nuclear genes together; (5) all DNA sequence data; and (6) a total combined analysis of all data. Support was estimated using bootstrap (200 replicates, 100 random addition sequences each) and Bremer support. Constraint trees for Bremer support analysis were constructed using AutoDecay 5.0

Table 3
GenBank accession numbers for sequences

	ArgK	EF-1α	Opsin	Wingless
Australian outgroups				
<i>H. (Gnathoprosopis) amicus</i>	DQ212137	DQ212154	DQ212170	DQ212120
<i>H. (Euprosopis) elegans</i>	DQ212138	DQ212155	DQ212171	DQ212121
Holarctic outgroups				
<i>H. (Hylaeus) leptcephalus</i>	DQ212139	DQ212156	DQ212172	DQ212122
<i>H. (Spatulariella) punctatus</i>	DQ212140	DQ212157	DQ212173	DQ212123
Japanese <i>Nesoprosopis</i>				
<i>H. (Nesoprosopis) globula</i>	DQ212141	DQ212158	DQ212174	DQ212124
Hawaiian <i>Nesoprosopis</i>				
<i>H. (Nesoprosopis) anthracinus</i>	DQ212142		DQ212174	DQ212125
<i>H. (Nesoprosopis) connectens</i>	DQ212143	DQ212159	DQ212175	DQ212126
<i>H. (Nesoprosopis) difficilis</i>	DQ212144	DQ212160	DQ212176	DQ212127
<i>H. (Nesoprosopis) hula</i>	DQ212145	DQ212161	DQ212176	DQ212128
<i>H. (Nesoprosopis) inquilina</i>	DQ212146	DQ212162	DQ212178	DQ212129
<i>H. (Nesoprosopis) kauaiensis</i>	DQ212147	DQ212163	DQ212179	DQ212130
<i>H. (Nesoprosopis) kokeensis</i>	DQ212148	DQ212164	DQ212180	DQ212131
<i>H. (Nesoprosopis) kona</i>	DQ212149	DQ212165	DQ212181	DQ212132
<i>H. (Nesoprosopis) longiceps</i>	DQ212150	DQ212166	DQ212182	DQ212133
<i>H. (Nesoprosopis) pele</i>	DQ212151	DQ212167	DQ212183	DQ212134
<i>H. (Nesoprosopis) pubescens</i>	DQ212152	DQ212168	DQ212184	DQ212135
<i>H. (Nesoprosopis) solaris</i>	DQ212153	DQ212169	DQ212185	DQ212136

Table 4
Sequences obtained for all genes.

gene	Length	Coding	Aligned intron	Informative total	Informative ingroup
ArgK	982	506	476	58	6
EF-1 α	1311	854	457	83	3
Opsin	695	500	195	49	7
wg	450	450	0	22	1
Total nuclear	3438	2310	1128	212	17
COI–COII	1201	1201 ^a	0 ^b	324	163
Total	4639	3511	1128	536	180

Units are base pairs.

^a Includes tRNA-Leu, 77 bp.

^b 32 bp noncoding region not included in analysis.

(Eriksson, 2003). Due to the extremely small number of informative nuclear characters (see Table 4), partitioned branch support was not calculated.

Rate comparisons were obtained by maximum likelihood using the “lscor” command in PAUP, using the mtDNA + morphology tree as found for the full 100-taxon data set (Magnacca and Danforth, 2006; Fig. 3). The GTR model was used with site-specific rates for 13 rate categories: ArgK, EF-1 α , opsin, and *wingless* third codon positions (first and second positions had virtually no variation); ArgK, EF-1 α , and opsin introns; and COI and COII first, second, and third positions. *Papilio* data (Reed and Sperling, 1999) includes about 200 additional bp of EF-1 α and all of COI and COII. *Columbicola* data (Johnson et al., 2003) is for 379 bp of COI and 347 bp of EF-1 α , overlap-

ping the 5' end of COI and partially overlapping the 3' end of EF-1 α in the *Hylaeus* data set.

3. Results

Full sequences were obtained for all taxa and genes (Table 4), except that EF-1 α could not be sequenced for *Hylaeus anthracinus*. This species was left out of the parsimony analysis for EF-1 α , and counted as missing data in all combined analyses. Due to high sequence concordance, alignment was trivial except in the ArgK intron for outgroup taxa. This region is much larger in the Hawaiian species than others in the sample: 9 of the 12 Hawaiian species had introns of 441 bp. Autapomorphic deletions of 17 (three separate deletions), 26, and 333 bp were present in *Hylaeus connectens*, *Hylaeus difficilis*, and *Hylaeus kauaiensis* respectively. By comparison, the intron in the Japanese *H. (Nesoprosopis) globula* was 211 bp, and only 81 bp in the Australian *H. (Gnathoprosopis) amicus*. Unlike introns in EF-1 α and opsin, which posed no alignment problems, a large part of the ArgK intron in the Australian species had no particular match with any segment in the non-Australian species. Since the closer Holarctic and Japanese outgroups aligned unambiguously with the Hawaiian species, the intron sequence was left as determined by MegAlign. However, the high degree of divergence compared to other intron sequences suggests it is likely the result of one or more separate insertion events. Two sets of repeated sequences (possibly microsatellites), one with TA repeats and the other with TG, were present in the opsin intron in

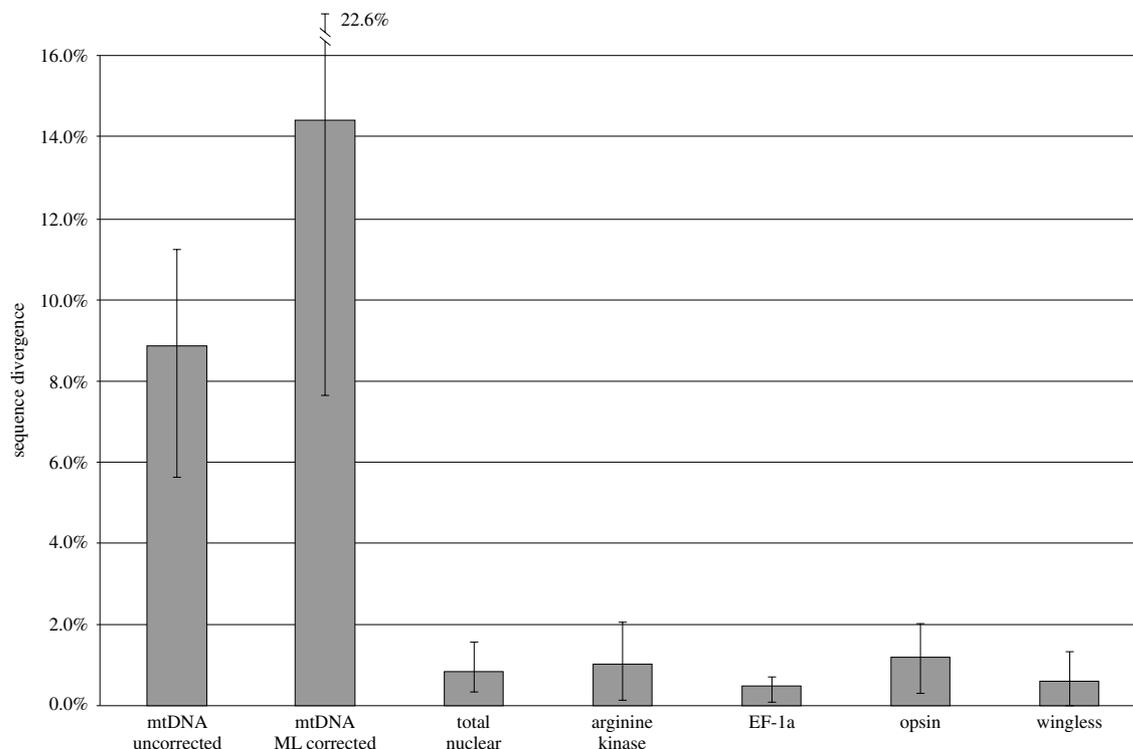


Fig. 1. Ingroup sequence divergence for all genes (uncorrected p distances except for ML-corrected mtDNA). Columns indicate average divergence across all species, error bars show maximum and minimum divergence.

all species except *Gnathoprosopis*. They were of constant length in other outgroup species, but varied considerably among the Hawaiian taxa.

Variation within the ingroup was extremely low for all nuclear genes, especially relative to the high degree of difference in the mitochondrial genes (Table 4; and Fig. 1). Pairwise divergence across the four nuclear genes (3438 aligned

bp) averaged 1.57%, and the highest value was 2.07%; the *wingless* sequences from *Hylaeus inquilina* and *Hylaeus kauaiensis* (two species that are clearly not the most closely related in the data set based on morphology and mtDNA sequences; see Fig. 3) were identical.

Most single data sets produced highly unresolved trees (Fig. 2). Both internal and terminal branch lengths are

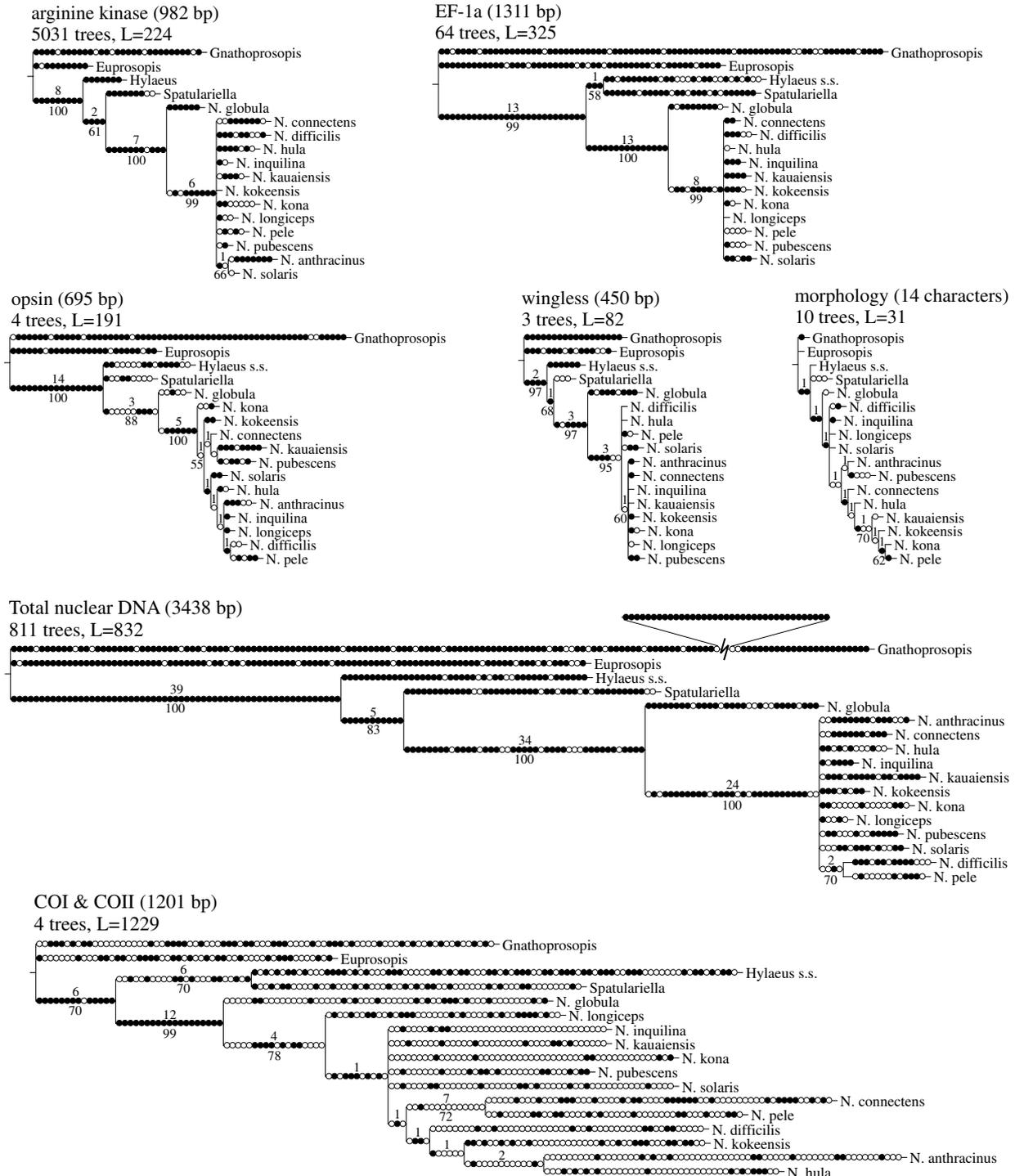


Fig. 2. Parsimony analysis of individual and combined nuclear genes, mitochondrial genes, and morphology. Strict consensus trees are shown; Bremer support and bootstrap values are above and below branches respectively. Circles indicate changes: Open circles are homoplasious, filled circles non-homoplasious (though possibly reversed).

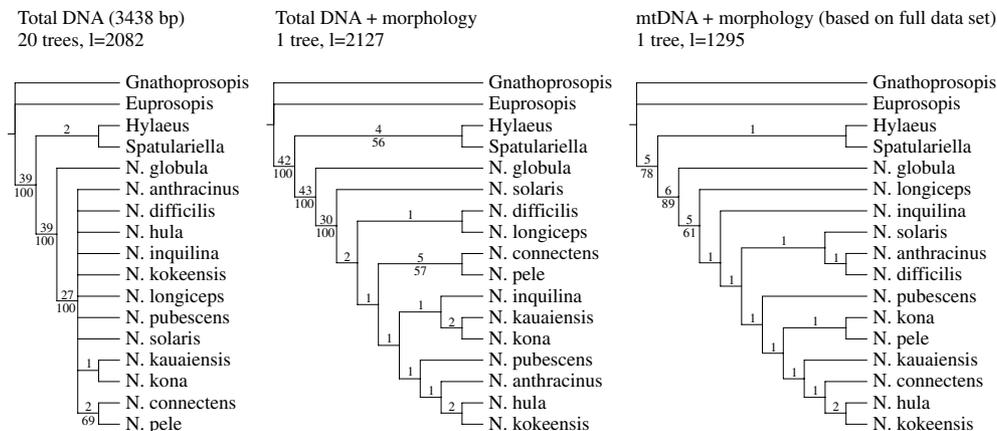


Fig. 3. Trees resulting from combined analyses. Changes not shown due to the large number. The right-most tree is based on that obtained by parsimony analysis of the complete 100-terminal data set for mtDNA and morphology (Magnacca and Danforth, 2006), and is that used for ML analysis of relative rates (Fig. 4). Bootstrap and Bremer support figures are likewise from the larger analysis.

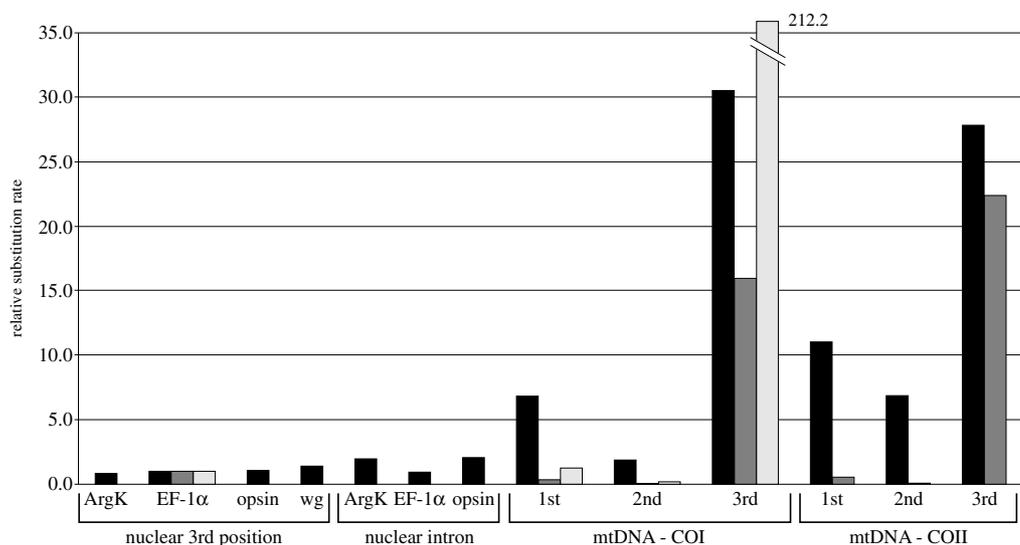


Fig. 4. Relative rates for data partitions, standardized at EF-1 α 3rd positions = 1.0. Rates for *Hylaeus* (black) are from a GTR + SSR analysis of the current set of species using the mtDNA + morphology tree (Fig. 3); data for *Papilio* (dark gray) are from Reed and Sperling (1999); data for *Columbicola* (light gray) are from Johnson et al. (2003). *Papilio* data includes about 200 additional bp of EF-1 α and all of COI and COII. *Columbicola* data is for 379 bp of COI and 347 bp of EF-1 α , overlapping the 5' end of COI and partially overlapping the 3' end of EF-1 α in the *Hylaeus* data set.

short, reflecting the slight variation. There were only 17 parsimony-informative (and 113 uninformative) differences among the Hawaiian species in the entire 3438 bp nuclear data set. Surprisingly, despite the low level of divergence, those differences that were shared between taxa were often in conflict or homoplasious. The opsin tree is relatively well-resolved, but all internal nodes have a branch length of 1, and the placement of *Hylaeus pele* is in serious conflict with morphological, mitochondrial, and ecological data that place it in the same species group as *Hylaeus kona* (Daly and Magnacca, 2003; Magnacca and Danforth, 2006). Combined analysis of all data sets resulted in a more resolved tree, but one that conflicts significantly with the previous phylogeny based on the full data set of 67 species and 100 terminals (Fig. 3). Moreover, analysis of the DNA evidence alone demonstrates that it provides little resolu-

tion, and much of the structure of the combined tree is due to the small morphological data set.

ML-calculated relative rates show the strong contrast between nuclear and mitochondrial genes (Fig. 4). The nuclear genes all evolve at approximately the same rate, with introns slightly faster than third positions in ArgK and opsin (though not in EF-1 α). Mitochondrial third positions evolve at about 30 times the nuclear rate, relatively faster than the rate found in *Papilio* butterflies (Reed and Sperling, 1999), though lower than that estimated for lice (Johnson et al., 2003). First and second positions in mtDNA (not shown for nuclear genes due to the near-total lack of differences) are also high. When the outgroups are included the mitochondrial rate drops to about seven times that of EF-1 α (not shown), a figure more in line with previous estimates (Lin and Danforth, 2004). However, since

mtDNA is already near saturation in the ingroup, ML estimates for amount of change may not be accurate at the greater distance.

4. Discussion

Although nuclear genes in general evolve much more slowly than mitochondrial genes (DeSalle et al., 1987; Lin and Danforth, 2004), the very low rate of divergence reported here was still surprising. For example, in *Heliconius* butterflies *wingless* evolved at about 0.6 times the rate of COII (both third positions only; *wingless* is the same fragment, COII includes an additional 371 bp 3' of the *Hylaeus* data set) within a single species, *Heliconius erato*, and overtakes it in sequence divergence at higher levels as base composition bias rapidly leads to saturation in COII (Brower and DeSalle, 1998). Yet in *Nesoprosopis*, *wingless* appears to be evolving more than an order of magnitude slower than COI and COII, at about the same rate as EF-1 α .

Comparison of relative rates of evolution among the nuclear genes is not particularly meaningful. The nuclear genes sequenced are thought to evolve at quite different rates (Brower and DeSalle, 1998; Friedlander et al., 1994; Mardulyn and Cameron, 1999), so the fact that they are almost uniformly slow, even in introns, implies that there has not been enough time for changes to build up in the faster genes. The small number of differences in the nuclear genes makes short-term random chance, rather than evolutionary characteristics of the genes, the overwhelming factor. This is particularly true when looking at the rates of different genes in pairwise comparisons of species, where some that are not sister species may have near-identical sequences, and having two differences rather than one appears as twice the amount of change. Nevertheless, two conclusions are inescapable: The mitochondrial and nuclear rates are vastly different, much more so than in most previous comparisons (Lin and Danforth, 2004); and the taxa constituting the Hawaiian clade are extremely closely related.

The fundamental problem with molecular phylogenetics in the Hawaiian bees is one of a lack of overlap in the utility of nuclear and mitochondrial genes. COI and COII—generally considered the slowest mitochondrial genes for non-synonymous changes (Simon et al., 1994; Wolstenholme and Clary, 1985)—are clearly evolving at an elevated rate, as evidenced by the high degree of difference between island populations and heteroplasmy within individuals (Magnacca and Danforth, 2006), to such a degree that mtDNA is barely useful in discerning the genetic history of a single species' haplotypes (in Fig. 4 it is worth noting that even though the mtDNA rate in *Columbicola* lice is much higher than *Hylaeus* for third positions, the reverse is true for first and second positions). Yet even the “fast-evolving” nuclear genes—e.g. *wingless*, and even introns—show so little variation that there is effectively no support for any relationships within the Hawaiian species. Although large numbers of

loci with low divergence can be combined to generate useful phylogenies of closely-related species (e.g., Jennings and Edwards, 2005), the high degree of homoplasy among the nuclear mutations present here suggest that such a method will not be useful for *Nesoprosopis*.

The very lack of variation in the nuclear genes supports a recent origin for the radiation in Hawaii, although the small amount of change also precludes dating using evolutionary rates. The high degree of conflict among nuclear DNA characters also supports the idea of a rapid radiation; the species groups share no clear genetic synapomorphies among them, and so that virtually all of the differences seen today must have evolved independently since that early radiation. Thus, the data presented here reinforces the conclusions based on the mtDNA/morphology tree—that the group originated and radiated on the island of Hawaii less than 700,000 years ago (Magnacca and Danforth, 2006). The combined weight of the various lines of evidence gives convincing support for a recent origin of *Hylaeus* in Hawaii.

Acknowledgments

We thank Yasuo Maeta of Tottori University and John Ascher of the American Museum of Natural History for supplying non-Hawaiian specimens, and Patrick O'Grady for comments on the manuscript. Funding came from an N.S.F. Doctoral Dissertation Improvement Grant to K.N.M.

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