

## Molecular Systematics and Adaptive Radiation of Hawaii's Endemic Damselfly Genus *Megalagrion* (Odonata: Coenagrionidae)

STEVE JORDAN,<sup>1,3</sup> CHRIS SIMON,<sup>1</sup> AND DAN POLHEMUS<sup>2</sup>

<sup>1</sup>Department of Ecology and Evolutionary Biology, University of Connecticut, Box U-3043, Storrs, Connecticut 06269, USA;  
E-mail: chris.simon@uconn.edu (C.S.)

<sup>2</sup>Department of Systematic Biology, MRC 105, Smithsonian Institution, Washington, D.C. 20560, USA; E-mail: bugman@mail.bishopmuseum.org

**Abstract.**—Damselflies of the endemic Hawaiian genus *Megalagrion* have radiated into a wide variety of habitats and are an excellent model group for the study of adaptive radiation. Past phylogenetic analysis based on morphological characters has been problematic. Here, we examine relationships among 56 individuals from 20 of the 23 described species using maximum likelihood (ML) and Bayesian phylogenetic analysis of mitochondrial (1,287 bp) and nuclear (1,039 bp) DNA sequence data. Models of evolution were chosen using the Akaike information criterion. Problems with distant outgroups were accommodated by constraining the best ML ingroup topology but allowing the outgroups to attach to any ingroup branch in a bootstrap analysis. No strong contradictions were obtained between either data partition and the combined data set. Areas of disagreement are mainly confined to clades that are strongly supported by the mitochondrial DNA and weakly supported by the elongation factor 1 $\alpha$  data because of lack of changes. However, the combined analysis resulted in a unique tree. Correlation between Bayesian posterior probabilities and bootstrap percentages decreased in concert with decreasing information in the data partitions. In cases where nodes were supported by single characters bootstrap proportions were dramatically reduced compared with posterior probabilities. Two speciation patterns were evident from the phylogenetic analysis. First, most speciation is interisland and occurred as members of established ecological guilds colonized new volcanoes after they emerged from the sea. Second, there are several instances of rapid radiation into a variety of specialized habitats, in one case entirely within the island of Kauai. Application of a local clock procedure to the mitochondrial DNA topology suggests that two of these radiations correspond to the development of habitat on the islands of Kauai and Oahu. About 4.0 million years ago, species simultaneously moved into fast streams and plant leaf axils on Kauai, and about 1.5 million years later another group moved simultaneously to seeps and terrestrial habitats on Oahu. Results from the local clock analysis also strongly suggest that *Megalagrion* arrived in Hawaii about 10 million years ago, well before the emergence of Kauai. Date estimates were more sensitive to the particular node that was fixed in time than to the model of local branch evolution used. We propose a general model for the development of endemic damselfly species on Hawaiian Islands and document five potential cases of hybridization (*M. xanthomelas*  $\times$  *M. pacificum*, *M. eudytum*  $\times$  *M. vagabundum*, *M. orobates*  $\times$  *M. oresitrophum*, *M. nesiotis*  $\times$  *M. oahuense*, and *M. mauka*  $\times$  *M. paludicola*). [Bayesian phylogenetics; Hawaii; local molecular clock; *Megalagrion* damselflies; species radiations.]

The study of adaptive radiation and speciation has advanced rapidly in recent years, especially for island taxa (Schluter, 2000). Even so, in a recent review of island arthropod evolution, Gillespie and Roderick (2002) concluded that our understanding of the interplay between isolation and time in the development of island biotas was limited. Although the broad outlines of this interplay can be sketched, specific data on the time required for speciation and radiation by particular organisms under particular conditions is lacking (but see, for example, Hormiga et al., 2003). As Gillespie and Roderick (2002) have described, dispersal that is nearly impossible for one organism is trivial for another, discouraging generalization. With the advent of molecular techniques, including methods for dating cladogenic events, we can now begin to generate specific estimates of speciation and radiation rates for taxa of various dispersal abilities on islands with different degrees of isolation. In this manner, we can clarify the details of community assembly on a variety of island types.

In an effort to address this important issue, we have used molecular data to investigate phylogeny and speciation within an endemic Hawaiian insect radiation. Hawaii and its *Megalagrion* damselflies offer an excellent

opportunity to explore the interaction of isolation and time in speciation and radiation. We have applied the local molecular clock of Yoder and Yang (2000) to give these explorations a temporal framework. The data set we have assembled includes multiple individuals from all available species. Thus, it meets the criteria of Barraclough and Nee (2001), who proposed that such phylogenetic data sets can illuminate the processes leading to speciation.

### Hawaii

Hawaii is known for its spectacular radiations of plants and animals. Many, such as the Hawaiian *Drosophila*, silverswords, and honeycreepers, are famous and classic examples of adaptation. Others, such as *Plagithmysus* long-horned beetles (Gressitt and Davis, 1969) and *Hyposmocoma* microlepidoptera (Zimmerman, 1978), are less well known but no less spectacular. This rich array of evolutionarily unique taxa has led to Hawaii's description as a natural laboratory for the study of evolution (Simon et al., 1984; Simon, 1987) where we have gained enriched understanding of evolutionary processes (e.g., Kaneshiro, 1974; Carlquist, 1980; Hoch and Howarth, 1993; Givnish et al., 1994; Tarr and Fleischer, 1995; Wagner and Funk, 1995; Craddock, 2000; Thacker and Hadfield, 2000; Barrier et al., 2001; Gillespie and Roderick, 2002; Paxinos et al., 2002). In particular, the

<sup>3</sup>Present address: Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Joseph Fourier, B.P. 53, 38041 Grenoble Cedex 9, France; E-mail: steve.jordan@ujf-grenoble.fr

phylogenies of many Hawaiian organisms have been useful in the study of speciation and adaptive radiation (Wagner and Funk, 1995; Schluter, 1998).

Hawaii's central importance to evolutionary studies stems from a confluence of several factors, most notably extreme, long-term isolation coupled with exceptional local variation in topography and climate. The Hawaiian chain is the product of a volcanic hot spot that remains stationary beneath the drifting Pacific tectonic plate. The main Hawaiian Islands (those with substantial subaerial volcanic mass, termed high islands) are thus arranged linearly, in chronological order (Fig. 1), with Kauai (5.1 million years [MY]) being the oldest major high island and Hawaii (0.43 MY) being the youngest (Carson and Clague, 1995). Although the Hawaiian Island chain has probably existed for 80–85 MY, there was a period between approximately 34 and 30 MYA when there were no emergent islands (Clague, 1996). Between 30 and 23 MYA, only low islands existed in the Hawaiian chain, offering no habitat to strictly high-island organisms. Since 23 MYA, there have always been high islands available to colonizing propagules (Clague, 1996). Taxa that became established on high islands since 23 MYA could have colonized new high islands as they became available, in theory progressing all the way to the current island of Hawaii; thus, 23 MY is the maximum possible

age of the Hawaiian high-island biota. However, Price and Clague (2002) modeled topographical change in the Hawaiian Islands over the past 32 MY and noted that before Kauai, high islands were small and distantly spaced, suggesting that most speciation has occurred within the extant main Hawaiian Islands.

It is difficult to exaggerate the biological isolation of the Hawaiian chain, which is roughly 3,800 km from the nearest continent (North America) and the nearest high islands (the Marquesas). No extant equivalent land mass has maintained such a substantial distance from continents or other high islands for so many millions of years (Clague, 1996). Gillespie and Roderick (2002) pointed out that adaptive radiation is likely to occur on extremely isolated islands such as Hawaii, where the speciation rate outstrips the immigration rate. In addition, as oceanic (newly created) islands, many resources in Hawaii were underexploited when the first colonizers arrived (Gillespie and Roderick, 2002).

The Hawaiian Islands are also the highest oceanic islands in the world, with the tallest summit, Mauna Kea, rising to 4,205 m. As with all such volcanic islands, the actual height of any given Hawaiian summit is constantly changing in response to erosion, volcanic deposition, and isostatic compensation. For example, increases in island height due to deposition of new lava are often offset by

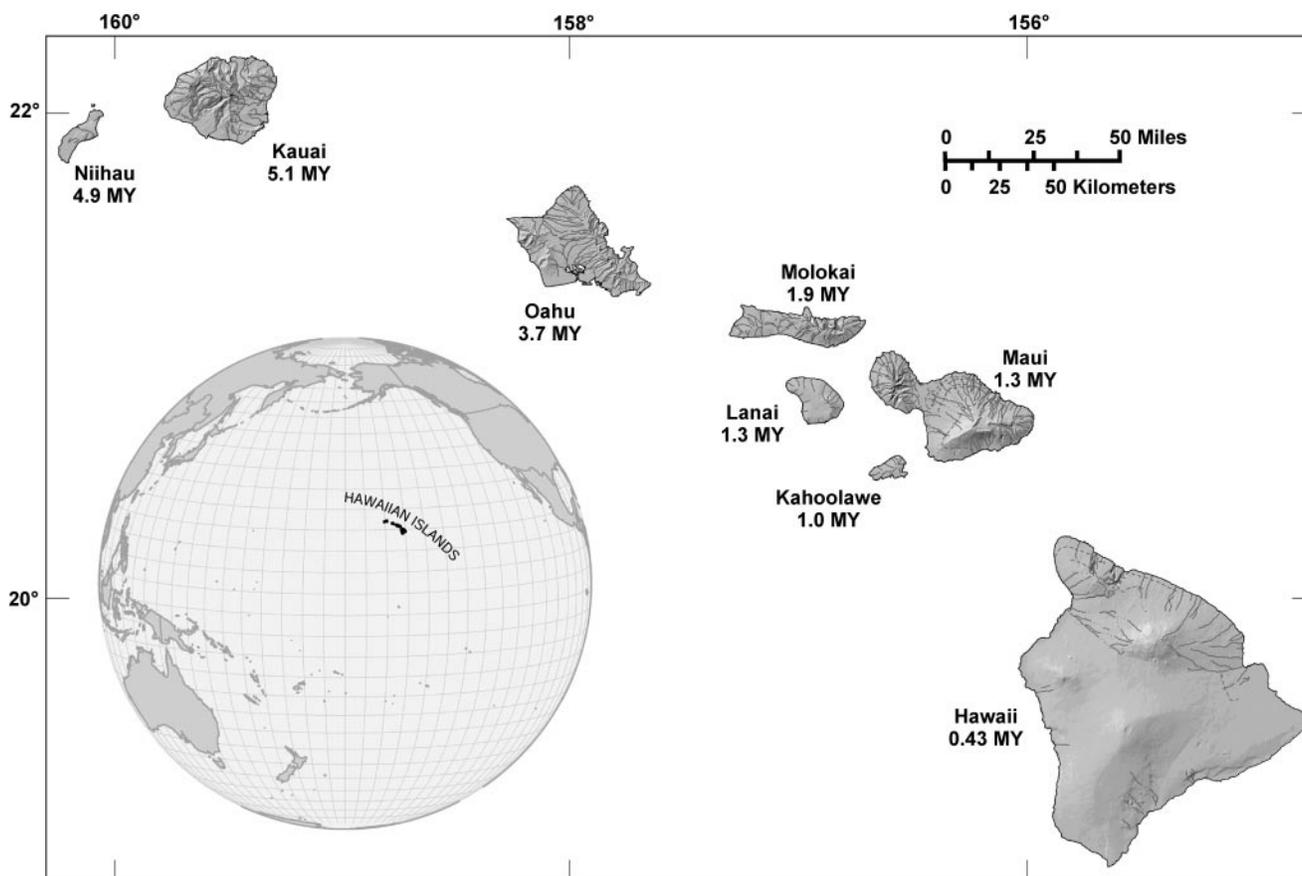


FIGURE 1. Map of the high Hawaiian Islands, showing K-Ar dates from Clague and Dalrymple (1987).

subsidence as the island's increased weight causes it to sink into the earth's mantle. Such subsidence rates are estimated to be as high as  $2.6 \pm 0.4$  mm/year (Ludwig et al., 1991). Similarly, older islands that lose material because of erosion tend to float upward in the mantle, maintaining a particular height range for long periods of geologic time. The surface effects of erosion are pronounced on the older islands, with precipitous gorges carved into their flanks and separated by sheer-sided ridges. In contrast, the younger, volcanically active islands have smooth, gentle slopes. Soils vary with island age, and younger volcanoes may have less surface water and fewer freshwater habitat types available than do older islands.

These extremes of topography combine with a predictable trade wind regime to produce a set of complex local microclimates within the Hawaiian Islands. Above 2,000 m, a consistent inversion in atmospheric temperatures blocks the upward rise of moisture carried in the trade winds (Hotchkiss et al., 2000). Although the exact elevation of this inversion varies on a daily basis and its mean elevation has varied over time, it currently acts to concentrate rainfall in a zone between 1,000 and 2,000 m elevation on the windward (northeastern) faces of the islands. As a result, average rainfall varies markedly by region, from  $>1,000$  cm/year on certain summits within the inversion zone to  $<20$  cm/year at topographically sheltered leeward sites near sea level. Further environmental complexity is also created by long-term variability in the lapse rate, a measure of the change in temperature that comes with increasing elevation (Hotchkiss et al., 2000).

#### Hawaiian Damselflies

Among the lesser known Hawaiian radiations is the endemic damselfly genus *Megalagrion*, which contains 23 recognized species (Polhemus and Asquith, 1996; Daigle, 1997; Polhemus, 1997). These species occupy as many breeding habitats as all the other damselflies in the world combined (Simon, 1987). *Megalagrion* is one of the most speciose insular damselfly radiations in the Pacific, rivaled only by the 30+ species in the Fijian genera *Nesobasis* and *Melanesobasis* (Donnelly, 1990).

*Megalagrion* are found on all the main Hawaiian Islands. Species richness and the level of endemism are directly correlated with island age. Kauai, the oldest of the current Hawaiian high islands, is the documented home of 12 species, 10 of which are endemic. Oahu has 8 species, 3 of which are endemic, the Maui Nui complex (Molokai, Maui, Lanai, and Kahoolawe) has 10 species, with 2 endemic, and the island of Hawaii has only 8 species, none of which are endemic.

The probable area of origin for ancestors of the genus *Megalagrion* is not known. Polhemus et al. (2000) noted that the damselfly fauna of Pacific islands east of the Tonga Trench and the Marianas Islands is represented by four other genera, all from the family Coenagrionidae: *Ischnura*, *Pseudagrion*, *Bedfordia*, and *Teinobasis*. The geographically closest of these to Hawaii are *Pseudagrion* and

*Bedfordia* reported from the Marquesas Islands. However, recent work has shown that the Marquesan taxon assigned to *Pseudagrion* should more properly be placed in *Bedfordia* and that *Bedfordia* as a whole is closely related to *Ischnura* (Polhemus et al., 2000). There are several *Ischnura* radiations in the eastern Pacific (Society Islands and Samoa), but insular Pacific *Teinobasis* are confined to Micronesia.

Selys-Longchamps (1876) offered the first description of a *Megalagrion* species, *M. xanthomelas*, although the genus was not described for 6 more years (McLachlan, 1883). At that time, McLachlan applied the name *Megalagrion* to only a few species in the modern genus, basing the generic name on wing characters later recognized as being too variable to have any taxonomic value (Perkins, 1913; Zimmerman, 1948). The first comprehensive monographic treatment of Hawaiian damselflies was done by Perkins, whose extensive collections dating from 1892 to 1901 formed the basis for his landmark work *Fauna Hawaiiensis* (Perkins, 1899, 1910).

Kennedy examined male genital characters of *Megalagrion* and published two articles on the genus (Kennedy, 1917, 1929). In one of these, provocatively titled "The origin of the Hawaiian Odonata fauna and its evolution within the islands," Kennedy (1929:979) posited that *Megalagrion* is so closely related to the Asian genus *Pseudagrion* that "—species of *Megalagrion* could be placed in [it] without hesitation, if found in the Orient." He concluded that "*Megalagrion* was introduced into the islands from Asia in early times, perhaps Eocene" (Kennedy, 1929:979). Unfortunately, this article contains little about the specific characters and methodologies employed to arrive at these conclusions, and the hypothesis of Eocene colonization is at odds with current geological knowledge.

A revision of the genus was carried out in 1948 by Zimmerman as a part of his remarkable achievement, *Insects of Hawaii*. He noted that affinities in *Megalagrion* were difficult to understand, in part because of the great variability found in traditionally useful zygopteran characters such as wing venation and color. He went so far as to note that "the wings on one side of a specimen may appear to belong to a different genus from those of the opposite side" (Zimmerman, 1948:350). Like Kennedy, Zimmerman (1948) wondered whether *Megalagrion* was closely allied to *Pseudagrion*, in part because of similarities noted among its members and some interesting adult and naiad specimens, putatively belonging to *Pseudagrion*, collected from the Marquesas Islands. He concluded by stating that "it would be most worthwhile to make a detailed comparison of *Megalagrion* with various species groups of *Pseudagrion* from diverse localities" (Zimmerman, 1948:347). Zimmerman also noted that the genus *Megalagrion* may be based more on location than morphology. Delimiting unique morphological characters to unambiguously define *Megalagrion* within the Coenagrionidae has proven challenging (Zimmerman, 1948; Polhemus, 1997), and morphological synapomorphies for the genus have not yet been described, although some candidate features

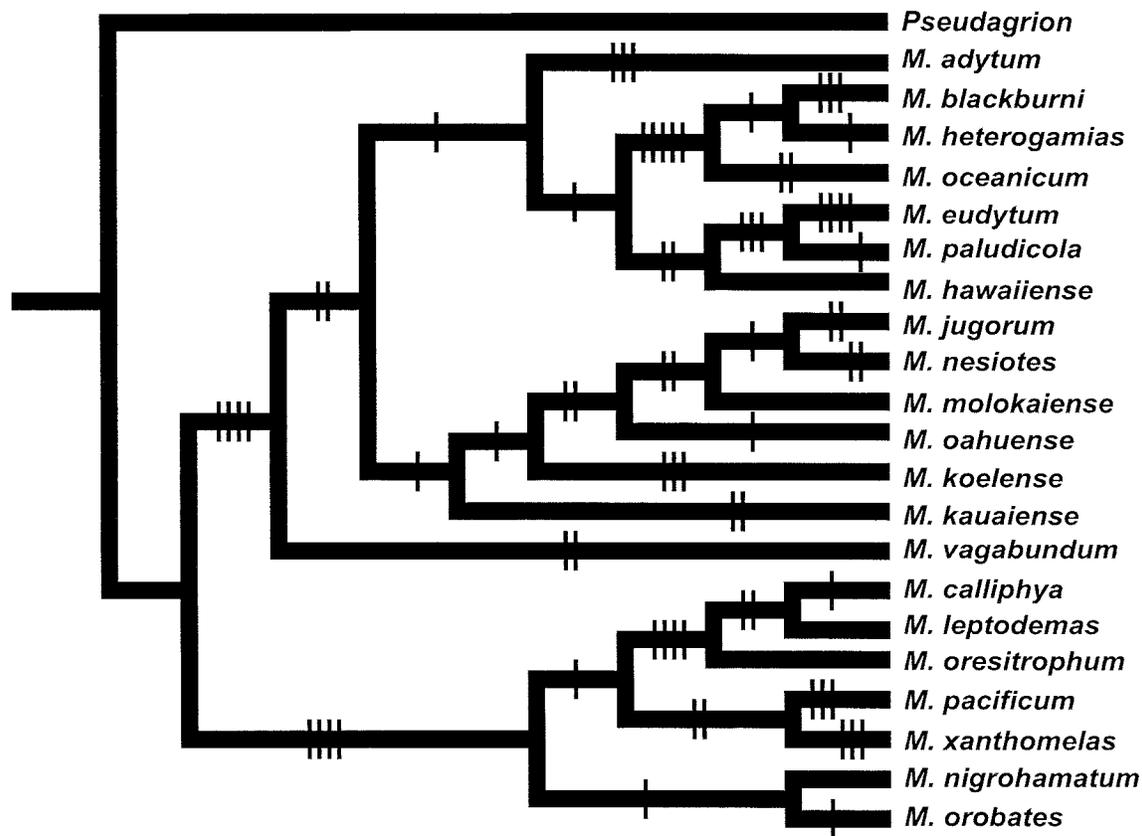


FIGURE 2. Hypothesis of *Megalagrion* phylogeny based on analysis of 22 morphological characters, from Polhemus (1997). Evolutionary steps are indicated by hash marks on branches.

have recently been identified (Polhemus, unpubl.). The lack of adequately defined morphological synapomorphies is not a problem confined to *Megalagrion*, being chronic throughout the Coenagrionidae as a whole.

Modern cladistic methods were brought to bear on the problem of *Megalagrion* phylogeny by Polhemus (1997), who also noted that morphological characters in *Megalagrion* are highly diversified and in some cases intraspecifically variable. Using 22 adult and larval characters, he generated a well-resolved tree that is in general harmony with the ideas of Zimmerman (1948). However, the influence of a small data set is apparent in this tree, where 14 of 20 ingroup nodes have only one or two supporting characters (Fig. 2). Polhemus (1997) used his cladistic hypothesis to study the evolution of gill morphology and habitat usage that accompanied this spectacular ecological radiation.

In the current study, we used two independent molecular data sets to reconstruct the phylogenetic history of *Megalagrion*. We conducted broad sampling of species within the genus and of individual populations within widespread species, comparing our results to previous hypotheses of *Megalagrion* phylogenetic relationships. Dates of divergence have been estimated for nodes of this tree, and these dates have been used in conjunction with the shape of the tree to explore speciation and adaptive radiation in *Megalagrion*.

## MATERIALS AND METHODS

### *Specimens Examined*

Nuclear and mitochondrial sequence data were collected from 64 individual damselflies (Table 1). Eight of these specimens were from outgroup species, and 56 were from 20 described *Megalagrion* species and 1 potentially new species. All species can be identified by male genitalia and female mesostigmal lamellae. At least two individuals were sequenced from each *Megalagrion* species to test for hybridization, sample mix-up, and/or laboratory contamination. Attempts were made to sample the 21 known extant species from every island where they occur. We were successful in obtaining these specimens, with the exception of Lanai Island populations of several widespread species and *M. williamsoni* from Kauai, which was only recently rediscovered. We also sampled individuals from populations that were known to harbor unique morphological traits, even if that led to more than one sample per island. The seven species that occur on multiple islands were sampled from at least two islands. *Megalagrion koelense* and *M. xanthomelas* were both sampled from five islands (Table 1). A separate study was focused on the phylogeography of *M. xanthomelas* and *M. pacificum* (Jordan, 2001).

Each specimen was given a unique number for this study (Table 1). Wings, abdomens, heads, and terminalia

TABLE 1. Specimens examined in this study. All collecting dates are in the 20th or 21st centuries.

No. specimens	Species	ID no.	Locale	Collection date	Elevation (m)	Permanent ownership <sup>a</sup>
2	<i>Megalagrion adytum</i>	A1, <sup>b</sup> A2	Kauai, Waialae–Mohihi Trail	19 May 95	1,280	BPBM
1	<i>M. blackburni</i>	B6 <sup>b</sup>	Molokai, Waikolu Valley	28 Aug 97	274	BPBM
1		B28	Maui, Makamakaole Stream, West Maui	16 Jul 98	182	BPBM
1		B33	Hawaii, South Hilo District, upper Paheehē Stream, near Akaka Falls	3 Mar 94	350	BPBM
1	<i>M. calliphya</i>	C2 <sup>b</sup>	Molokai, Waikolu	28 Aug 97	274	BPBM
1		C14	Maui, East Maui, upper Hanawi Stream	11 Nov 92	890	BPBM
1		C30	Hawaii, Hawaii Volcanoes National Park, Olaa Forest	23 Jul 96	500	HVNP
1	<i>M. eudytum</i>	E1 <sup>b</sup>	Kauai, Kokee, Koaie	3 Aug 97	1,097	BPBM
1		E8 <sup>b</sup>	Kauai, large stream draining Kanaele Swamp	22 Jul 98	609	BPBM
1	<i>M. hawaiiense</i>	Hw1 <sup>b</sup>	Oahu, Mt. Kaala	14 Aug 97	1,160	BPBM
1		Hw29 <sup>b</sup>	Oahu, Koolau Mountains, Waikane Stream	2 Jun 95	213	BPBM
1		Hw2 <sup>b</sup>	Molokai, Waikolu	28 Aug 97	274	BPBM
1		Hw81 <sup>b</sup>	Maui, West Maui, Mt. Eke	23 May 97	1,310	BPBM
1		Hw59 <sup>b</sup>	Hawaii, Onomea Stream above garden	25 Jul 98	55	BPBM
1	<i>M. heterogamias</i>	Ht1 <sup>b</sup>	Kauai, Kokee, Koaie Stream	2 Aug 97	1,188	BPBM
1		Ht22	Kauai, Kanaele Swamp	22 Jul 98	609	BPBM
1	<i>M. kauaiense</i>	Ka1 <sup>b</sup>	Kauai, Makaleha Mountains, Pohakupili	11 Aug 96	792	BPBM
1		Ka2	Kauai, Mt. Kahili, on trail to radio tower	10 Aug 96		BPBM
1	Sp. nov.	Ka4 <sup>b</sup>	Kauai, road to Kanaele Swamp	22 Jul 98	550	BPBM
1	<i>M. koelense</i>	KL50 <sup>b</sup>	Hawaii, Hawaii Volcanoes National Park, Olaa Forest	8 Aug 00	1,070	HVNP
1		KL10 <sup>b</sup>	Maui, West Maui mountains, Puu Kukui, summit trail	13 Aug 97	1,615	BPBM
1		KL5	Molokai, TNC Kamakou Preserve, Hanalilolilo	5 Dec 96	1,160	HVNP
1		KL94 <sup>b</sup>	Lanai, Mt. Haalelepaakai, Lanaihale in <i>Freyinetia arborea</i>	2 May 93	1,018	BPBM
1		KL6	Oahu, North Halawa Valley, along stream	26 Dec 91	300	BPBM
1		KL25 <sup>b</sup>	Oahu, Mt. Kaala, Dupont Trail, in <i>Freyinetia</i>	20 Aug 00	1,121	HVNP
2	<i>M. leptodemas</i>	L1, <sup>b</sup> L2	Oahu, Maakua Stream	29 Jul 98	182	BPBM
1	<i>M. mauka</i>	Hw40 <sup>b</sup>	Kauai, Mt. Waialeale	17 May 95	1,480	BPBM
1		M1 <sup>b</sup>	Kauai, Kalalau Trail, mile 1	23 Jul 98	35	BPBM
1	<i>M. nesiotēs</i>	Ns3 <sup>b</sup>	Maui, East Wailua Iki Stream	14 May 95	380	BPBM
1		Ns4		20 Jul 94	381	BPBM
1	<i>M. nigrohamatum nigrohamatum</i>	Nh1 <sup>b</sup>	Maui, West Maui, Iao Valley	6 May 94	335	BPBM
1		Nh4	Molokai, Waikolu	28 Aug 97	274	BPBM
1	<i>M. n. nigrolineatum</i>	NL3 <sup>b</sup>	Oahu, Schofield–Waikane Trail	19 May 91	518	BPBM
1	<i>M. oahuense</i>	Oa4 <sup>b</sup>	Oahu, Konahua nui	13 Aug 97	963	BPBM
1		Oa7	Oahu, North Halawa Valley	8 Dec 93	303	BPBM
1	<i>M. oceanicum</i>	Oc1 <sup>b</sup>	Oahu, Kaipapau Stream	20 Jun 93	304	BPBM
1		Oc2	Oahu, Koolau Mountains, Koloa Gulch above Laie	19 Jan 95	274	BPBM
1	<i>M. oresitrophum</i>	Op3	Kauai, Kawaikoi Stream	1 Aug 97	1,066	BPBM
1		Op9 <sup>b</sup>	Kauai, Kanaele Swamp	22 Jul 98	609	BPBM
3	<i>M. orobates</i>	Ob1, <sup>b</sup> Ob2, <sup>b</sup> Ob3	Kauai, Upper Moalepe Stream	10 Nov 94	246	BPBM
1	<i>M. pacificum</i>	Pc8 <sup>b</sup>	Molokai, Wailau Valley	18 Jul 98	0	BPBM
1		Pc14 <sup>b</sup>	Maui, Haipuaena Stream	14 May 95	182	BPBM
1		Pc21 <sup>b</sup>	Hawaii, tributary to north branch Maili Stream	31 Dec 98	515	BPBM
1	<i>M. paludicola</i>	Pa1 <sup>b</sup>	Kauai, Alakai Swamp, in bog near Kilohana	25 Jul 95	1,125	BPBM
1		Pa2	Kauai, Kanaele Swamp	22 Jul 98	609	BPBM
1	<i>M. vagabundum</i>	V1 <sup>b</sup>	Kauai, Kokee, Kauaiikina	7 Aug 97	1,036	BPBM
1		V3	Kauai, Koaie Stream	3 Aug 97	1,130	BPBM
1	<i>M. xanthomelas</i>	X10 <sup>b</sup>	Oahu, Tripler Army Medical Center	11 Sep 97	79	BPBM
1		X22 <sup>b</sup>	Lanai, pipeline seepage in lower Maunalei Gulch	26 Apr 95	45	BPBM
1		X54	Molokai, Kalaupapa fish pond	18 Jan 98	0	BPBM
1		X74	Maui, Papaka Kai, Cape Hanamanioa, easternmost anchialine pool	9 Oct 99	0	HVNP
1		X81 <sup>b</sup>	Hawaii, South Hilo, Keaukaha, Kealoha Beach Co. Park	29 Apr 00	0	HVNP

(Continued on next page)

TABLE 1. Continued

No. specimens	Species	ID no.	Locale	Collection date	Elevation (m)	Permanent ownership <sup>a</sup>
1	<i>Agrionemis exsudans</i>	Ae4	Fiji, Viti Levu: Navai V., Navai C.	26 Oct 98	667	KSJC
1	<i>Argia sedula</i>	As1	Texas, Upper Nueces River	2 May 88	245	DAPC
1	<i>Enallagma geminatum</i>	Eg1	Connecticut, Swan Lake, Univ. Connecticut	28 May 98	200	SDJC
1	<i>Ischnura aurora</i>	Ia38	Fiji, Viti Levu: 34.5 km west of Suva, Stream n/a	20 Oct 98	0	KSJC
1	<i>Ischnura heterosticta</i>	Ih23	Fiji, Vanua Levu: Saivou V., Seaqaqa R.	2 Nov 98	273	KSJC
1	<i>Pseudagrion civicum</i>	Psc1	Irian Jaya, Kopi River and tributary, north of Timika	18 Jan 97	106	BPBM
1	<i>Pseudagrion pacificum</i>	Psep1	Fiji, Viti Levu: 30.2 km north of Lautoka V., Stream n/a	25 Oct 98	76	KSJC
1	<i>Pseudagrion palauense</i>	PsePal1	Palau, Babeldaub Is., upper Ngerimel River, above reservoir	12 Aug 99	30	BPBM

<sup>a</sup>BPBM = Bernice P. Bishop Museum, Honolulu, Hawaii; HVNP = Pacific Island Ecosystems Research Center, U.S. Geological Survey, Hawaii Volcanoes National Park, Hawaii; KSJC = Kristen Jordan collection; SDJC = Steve Jordan collection; DAPC = Dan Polhemus collection.

<sup>b</sup>The 36 individuals forming the principal data set for this study.

have been preserved in individual vials. These voucher specimens will be deposited with the Bernice P. Bishop Museum in Honolulu, Hawaii, and the Kilauea Field Station, Pacific Island Ecosystems Research Center, U.S. Geological Survey, Hawaii Volcano National Park, Hawaii. Extracted DNA will remain in the care of S.J. until these museums develop suitable facilities to house it.

Eight species of coenagrionid damselflies were included as outgroups, although we initially worked to sequence more. We attempted to include representatives of all Oceanic and Pacific rim damselfly genera that could be the sister taxon to *Megalagrion*, including *Nesobasis* and *Agriocnemus* from Fiji, *Teinobasis* from Palau and Papua New Guinea, *Bedfordia* from the Marquesas, *Pseudagrion* from Irian Jaya, Palau, and Fiji, and *Ischnura* from Fiji. We placed particular emphasis on *Pseudagrion* to address Zimmerman's (1948) hypothesis that it represents the sister group. We also sampled *Argia* from Costa Rica, Texas, and Colorado and *Enallagma* from Connecticut.

#### DNA Extraction, Amplification, and Sequencing

DNA was extracted from damselfly thoracic muscles using either a standard phenol/chloroform method (Sambrook et al., 1989) or a Qiagen DNeasy Tissue Kit. We generated roughly 1,300 base pairs (bp) of DNA sequence data from three mitochondrial protein-coding genes (cytochrome oxidase II [COII], A6, A8) and two mitochondrial tRNA genes (lysine and aspartic acid), which we expected to show informative variation within this genus (Simon et al., 1994).

Mitochondrial DNA (mtDNA) PCR and sequencing were accomplished using two primer pairs. These primers have been named with reference to the *Drosophila yakuba* entire mtDNA genome sequence from GenBank (accession X03240; Clary and Wolstenholme, 1985) as suggested by Simon et al. (1994). The first primer pair amplifies the COII gene. Primer C2-J-3102 (5'-AA ATG GCA ACA TGA GCA CAA YT-3') spans the COII start codon and was developed for this study. Primer TK-N-3773 (5'-GAGACCAGTACTTGCTTCAGTCATC-3') sits in the lysine tRNA and is a modification of TK-N-

3785 (Liu and Beckenbach, 1992) developed by J. Fetzner (Brigham Young University). The second primer pair was developed for this study and spans the tRNAs, A8, and the 5' half of A6. Primer C2-J-3711 (5'-CAC AGA TTY ATR CCY ATY AC-3') is in the COII gene, near the 3' end. Primer A6-N-4379 (5'-CT TAA TCA TAA TGG TAA DGC TA-3') sits near the middle of the A6 gene. PCR for the mtDNA included 35 cycles of three steps each: (1) 30 sec at 94°C (denaturing), (2) 60 sec at 50°C (annealing), and (3) 60 sec at 72°C (extension).

We also sequenced approximately 1,000 bp of the nuclear elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) gene. Again, we used two primer pairs for most taxa. All four primers were designed for this study. We have named these primers according to the number of their 3' base in the *Drosophila melanogaster* EF-1 $\alpha$  sequence in GenBank (accession X06869; Hovemann et al., 1988). The first primer pair consists of EF1-F-2361 (5'-Y GGM CAC AGR GAT TTC ATC AA-3') and EF1-R-2765 (5'-GG TCG RCT RGG HGG MAG AAT-3'). The second pair consists of EF1-F-2652 (5'-TTY GTW CCV ATC TCM GGC TGG CA-3') and EF1-R-3093 (5'-CC AGG RTG GTT RAG CAC RAT GA-3'). PCR for the EF-1 $\alpha$  gene included 36 cycles of three steps each: (1) 30 sec at 94°C, (2) 60 sec at 62°C, and (3) 60 sec at 72°C.

Several other EF-1 $\alpha$  primers were needed for difficult taxa, including primers M44-1 and rcM53-2 from Cho et al. (1995) and two primers designed by F. Carle (Rutgers University) (E2F-2583: 5'-CT CGT TTY GAR GAA ATY AAG AAG GAA GT-3'; E2R-2921: 5'-TC AAC RGA YTT WAC YTC AGT-3').

Cycle sequencing of PCR products was carried out using Big Dye terminators and standard conditions. Every PCR product was sequenced with both of the primers used to create it. Sequences were visualized on an ABI 377 sequencer. All autapomorphies and nonsynonymous mutations were verified by rechecking the original chromatograms.

To identify possible contamination, extraordinary results were checked by gathering the data again. Any individual damselfly that appeared to be carrying DNA from another damselfly species had its DNA extracted,

amplified, and sequenced a second time, in strict temporal isolation from the first.

### *Phylogenetic Analysis*

*Alignment and sequence characteristics.*—Data were aligned first by ClustalX (Thompson et al., 1997). Alignments were inspected by eye and adjusted using information on codon position and amino acids. Nucleotide sites in EF-1 $\alpha$  that were potentially polymorphic were coded with each possible base. Base frequency homogeneity was tested using PAUP\* (Swofford, 1998). We ran this test on the mtDNA and the EF-1 $\alpha$  data partitions separately, first including all sites and then including only variable sites.

*Data set optimization.*—To speed computation time, each data set was trimmed of individuals that were redundant. We first examined the data for potential long branch attraction by performing a minimum evolution search with maximum likelihood (ML) distances calculated under a general time reversible (GTR) model that was corrected for among site rate variation (ASRV) using a combination of invariant sites and a discrete approximation of the gamma distribution (GTR+I+ $\Gamma$ ). When the internal branches of a named species were longer than the branch supporting its monophyly, all members of that species were retained for the full analysis, in spite of bootstrap support for species monophyly. Next, we considered bootstrap support for species monophyly. Four pairs of individual damselflies had identical sequences for the entire mtDNA and nuclear regions, and one member of each pair was deleted. We let a single damselfly represent every named species whose individuals had either identical sequences or >90% maximum parsimony (MP) bootstrap values for both the mtDNA and the EF-1 $\alpha$  data. These initial MP bootstrap values were obtained by analyzing all nonidentical ingroup sequences from each data partition (1,000 pseudoreplicates, two random addition sequence replicates, TBR branch swapping, MAXTREES = 2). We also removed four individuals from the data set that did not have complete data for one of the analyzed loci. The monophyly of these four individuals with conspecifics was confirmed in the initial MP analysis, and all species remained represented in the data set after removal of the four individuals. This initial culling procedure resulted in a data set of 36 *Megalagrion* individuals (Table 1).

After some phylogenetic searching, it became clear that data from at least two individuals (Pc21 and E8) were misleading because of likely introgressive hybridization. Nuclear DNA, morphological identification, and mitochondrial data for these damselflies were in conflict, so these two individuals were removed from the data set for some analyses, resulting in a data set of 34 individuals.

*Maximum likelihood.*—Every ML search in this study was conducted in the following manner using PAUP\*. We first selected the best model for each data partition using the methods of Frati et al. (1997) and Buckley et al. (2002) to compare 16 different likelihood models using likelihood ratio tests (LRTs) and the Akaike infor-

mation criterion (AIC) to select the simplest model that is not significantly different from the best-fitting model. Utilization of this simplest model reduces the error of estimated parameters (Swofford et al., 1996) and decreases computing time. In all ML searches, we estimated appropriate ML model parameters using the data partition of interest and a MP topology. These estimated parameter values were fixed, and a full heuristic ML search was conducted. All heuristic searches in this study were run with 10 random addition sequence replicates, retaining all minimal trees, and TBR branch swapping unless otherwise noted. After this first ML search was completed, we reestimated model parameters on the ML tree and used these new values to search again. We repeated this process until the new search found the same topology as the previous search and all parameter estimates were identical.

Using either the 34- or 36-individuals data set, we performed ML analyses on several data partitions, including COII and tRNAs combined, A6 and A8 combined and separate, all mtDNA combined, EF-1 $\alpha$  alone, and mtDNA and EF-1 $\alpha$  data combined. COII and tRNAs were combined because they are contiguous and the tRNA region is exceptionally short (150 bp).

We also implemented ML bootstrap searches on several of the data partitions, using the previously selected appropriate models. Again, ML parameter values were fixed under the appropriate model using the MP topology. We carried out 200 pseudoreplicates using heuristic searches with one random addition sequence replicate and MAXTREES = 1. DeBry and Olmstead (2000) used simulations to show that bootstrap values generated when one tree is retained produce results that are indistinguishable from those obtained when all minimal trees are retained.

The mtDNA data were tested for conformity to a molecular clock by conducting a heuristic search of 36 individuals under a GTR+I+ $\Gamma$  model, with molecular clock constraints. The likelihood of the resulting topology was compared with the likelihood of the same topology without the clock enforced using an LRT and  $df = 34$  (Felsenstein, 1983; Lewis, 1998).

*Bayesian analysis.*—Implementation of traditional ML methods in phylogenetics can be time consuming. The result of these methods represents only a point estimate of phylogeny unless even more time-intensive bootstrapping procedures are undertaken (Lewis, 2001). Fortunately, the use of ML models within a Bayesian framework is becoming more accessible to systematists (Larget and Simon, 1999; Huelsenbeck et al., 2000; Simon and Larget, 2000; Lewis, 2001). We implemented a Bayesian phylogenetic analysis using the MrBayes software of Huelsenbeck and Ronquist (2001). We used the GTR+I+ $\Gamma$  model for all analyses and ran four Markov Chain Monte Carlo (MCMC) chains, three heated and one cold. To assess the coverage of tree space, we performed two MCMC runs for each analysis, the first of 250,000 steps and the second of 1 million steps. These runs started from different random points in parameter space. All sample points that occurred before stationarity

of negative log likelihood ( $-\ln L$ ) scores was achieved were discarded as part of a burn-in period (Huelsenbeck and Ronquist, 2001). If the trees with the highest posterior probability for each of these runs agreed, we did not initiate any more runs. These Bayesian analyses were run for the 36 nonredundant individuals using the combined mtDNA data set and the EF-1 $\alpha$  data set. They were also run for the combined mtDNA and nuclear data set, using 34 individuals.

*Data partition congruence.*—Many authors have discussed the importance of data set congruence in phylogenetic studies using combined data (e.g., Bull et al., 1993; Chippindale and Wiens, 1994; Wiens, 1998). Initial data exploration using ML trees and the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) revealed that, as expected, all mtDNA partitions were congruent.

To remove a known source of bias, SH tests assessing congruence of mtDNA and EF-1 $\alpha$  data were run using the 34-specimen data set, which did not contain two individuals displaying signs of introgressive hybridization. Assumptions of this test are best met when the body of tested trees is thought to contain the ML topology for the tested data set (Goldman et al., 2000). We first compared 432 topologies, including the mtDNA ML tree, the 22 mtDNA MP trees, the EF-1 $\alpha$  ML tree, and the 408 EF-1 $\alpha$  MP trees. These trees were tested using two data sets: mtDNA data alone and EF-1 $\alpha$  data alone. Appropriate ML model parameters were estimated and fixed using an MP topology to save time. Based on the results of this initial SH test, we ran the test again, this time using fewer trees and estimating model parameters for each tested topology. In this case, the 15 tested topologies included the mtDNA ML tree, 5 of the 22 mtDNA MP trees, the EF-1 $\alpha$  ML tree, 5 of the 408 EF-1 $\alpha$  MP trees, the combined mtDNA–EF-1 $\alpha$  ML and MP trees, and the morphological topology of Polhemus (1997). These trees were compared using appropriate models and three data sets: mtDNA data, EF-1 $\alpha$  data, and combined mtDNA and EF-1 $\alpha$  data.

*Rooting.*—All of the above analyses were run with ingroup taxa only, for several reasons. First, for the mtDNA and EF-1 $\alpha$  data, preliminary model-corrected analyses showed that all outgroup branches were more than twice as long as the longest ingroup branches (inset, Fig. 3), and some were much longer. We did not analyze the outgroups with the ingroups to avoid negative effects on ingroup topology due to long branch attraction, a result we found to be possible in data exploration (Jordan, unpubl.). Second, for the EF-1 $\alpha$  data, the intron regions were not alignable among genera. Exclusion of the introns in a simultaneous analysis of ingroup and outgroup taxa would have resulted in the loss of most of the informative ingroup characters.

To root the ML topologies for the mtDNA data and the EF-1 $\alpha$  data, constrained ML bootstrap analyses were run. In these analyses, the best ML ingroup topology was set as a backbone constraint in PAUP\*. Outgroup taxa were added to this constrained ingroup, and an ML bootstrap search of 100 pseudoreplicates was con-

ducted (heuristic searches of one random addition sequence replicate, MAXTREES = 1). In this search, the ML relationships of the ingroup taxa were preserved but the outgroups were free to attach to any ingroup branch. All mtDNA data and EF-1 $\alpha$  exons were analyzed in this fashion. The combined nuclear and mitochondrial data were also analyzed in this manner, but with 200 bootstrap pseudoreplicates.

*Dating.*—An LRT revealed that evolution of the mtDNA data did not conform to a molecular clock ( $P = 0.001$ ,  $\chi^2 TS = 65$ , 34 df). Therefore, we used the local clock of Yoder and Yang (2000) to estimate branch lengths of the mtDNA ML topology. We used four separate scenarios of branch length variation. The first three scenarios allowed two possible rates and assigned the second rate to two, six, and nine branches, respectively (Fig. 4). The fourth scenario allowed three possible rates, assigning one rate to 2 branches, another to 27 branches, and the third to the remaining branches (Fig. 4). Under these scenarios, branches were assigned to rate classes based on visual inspection of their lengths on the unclocked mtDNA ML topology (Fig. 5a). A GTR+ $\Gamma$  model with a local clock was implemented using PAML (Yang, 2000). We did not use the EF-1 $\alpha$  topology for dating purposes because it lacked resolution. However, to examine the effect of an alternate rooting on age estimates of nodes, we reran the dating procedure on the mtDNA data and topology using the rooting suggested by the EF-1 $\alpha$  and combined data analyses.

The dates of nine nodes were fixed (one at a time) following the procedure of Yoder and Yang (2000). These dates were calculated using the procedure of Fleischer et al. (1998), who pointed out that the known ages of the Hawaiian Islands can be combined with phylogenetic information to fix certain cladogenic events in time. If a monophyletic group shows a comblike topology that is congruent with a pattern of radiation down the island chain, the ages of single island taxa are assumed to be no older than the island on which they are found. The nodes were fixed with the dates shown in Figure 4.

## RESULTS

### Sequences

Mitochondrial DNA and nuclear EF-1 $\alpha$  sequence data were obtained for 56 *Megalagrion* and 8 outgroup individuals (Table 1). Five genera were represented among the eight outgroup species sequenced (Table 1). EF-1 $\alpha$  sequences were not obtained from *Nesobasis*, *Teinobasis*, or *Bedfordia*; therefore, these genera were not included in the analysis. Sequences are available from GenBank (accessions AY179038–AY179165) and the aligned Nexus data file is available from TreeBase ([www.treebase.org](http://www.treebase.org)).

The final alignment, including outgroups, was 2,326 bp long, including 1,287 mtDNA characters (392 ingroup parsimony informative) and 1,039 EF-1 $\alpha$  characters (79 ingroup parsimony informative). The ingroup alignment was unambiguous for mitochondrial and nuclear data. Six pairs of individuals had identical

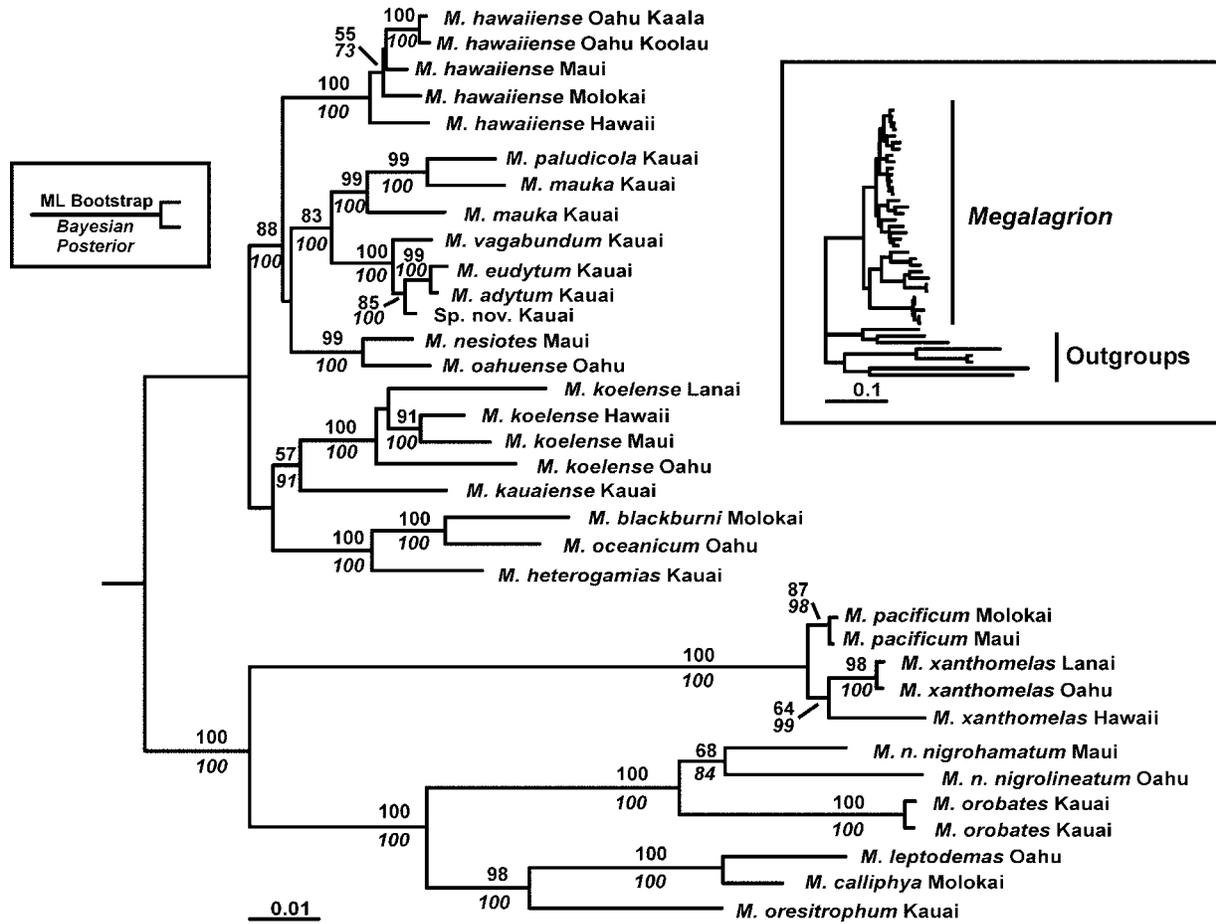


FIGURE 3. Maximum likelihood phylogram of *Megalagrion* damselfly species relationships based on a combined molecular analysis of 2,326 bp of nuclear EF-1 $\alpha$  and mtDNA sequence data, with nodal support indicated. A full heuristic ML analysis was performed under the GTR+I+ $\Gamma$  model, with starting parameters estimated from an MP topology. This search was repeated with parameters reestimated from the ML topology. Numbers above the line are ML bootstrap values based on 200 bootstrap pseudoreplicates. Numbers below the line are Bayesian posterior probabilities based on a 1 million step MCMC analysis. A node is shown as resolved if its bootstrap values is  $\geq 55$  and its posterior probability is  $\geq 65$ . *Inset*: Phylogram of combined data analysis with outgroups included. Ingroup topology was constrained to that of the tree in the main panel, but outgroups were free to attach to any branch. EF-1 $\alpha$  introns were excluded in this outgroup analysis.

mtDNA sequences. Seven pairs, four triplets, and one quadruplet had identical EF-1 $\alpha$  sequences. For the ingroup, A/T bias of the mtDNA data was 72%, but the data exhibited stationarity of base frequencies among taxa for all nucleotide sites ( $P = 1.0$ ) and for variable sites analyzed alone ( $P = 1.0$ ). Ingroup EF-1 $\alpha$  data showed an A/T bias of 52%. Again, base frequency stationarity was not violated for all sites ( $P = 1.0$ ) or for variable

sites ( $P = 1.0$ ). Uncorrected ingroup genetic distances ( $p$ ) were as high as 0.14 for the mtDNA and 0.06 for the EF-1 $\alpha$  data. GTR+I+ $\Gamma$  corrected distances ranged up to 0.25 for the mtDNA and 0.06 for the EF-1 $\alpha$  data. Only four EF-1 $\alpha$  sites (from four separate individuals) were polymorphic. Appropriate models and estimated parameter values for ML analyses are listed for each data partition in Table 2.

TABLE 2. Appropriate models and estimated model parameters for data partitions. These partitions were analyzed with the 36-specimen data set specified above, except for the combined mtDNA and EF-1 $\alpha$  analysis, which used the 34-specimen set. The last column allows a comparison of among-site rate variation using estimates of the gamma shape parameter  $\alpha$  under a GTR+ $\Gamma$  model.

Partition	Model	−lnL	Transition: transversion ratio	$\alpha$	I	$\alpha$ without I
COII tRNA	GTR+I+ $\Gamma$	3835.37		1.04	0.60	0.16
A6/A8	HKY+I+ $\Gamma$ <sup>a</sup>	9946.84	3.55	0.63	0.58	0.31
All mtDNA	GTR+I+ $\Gamma$	7017.11		1.21	0.56	0.18
EF-1 $\alpha$	GTR+ $\Gamma$	2496.69		0.25	na	0.25
Combined data	GTR+I+ $\Gamma$	9829.75		0.55	0.56	0.15

<sup>a</sup>HKY = Hasegawa-Kishino-Yano model (Hasegawa et al., 1985).

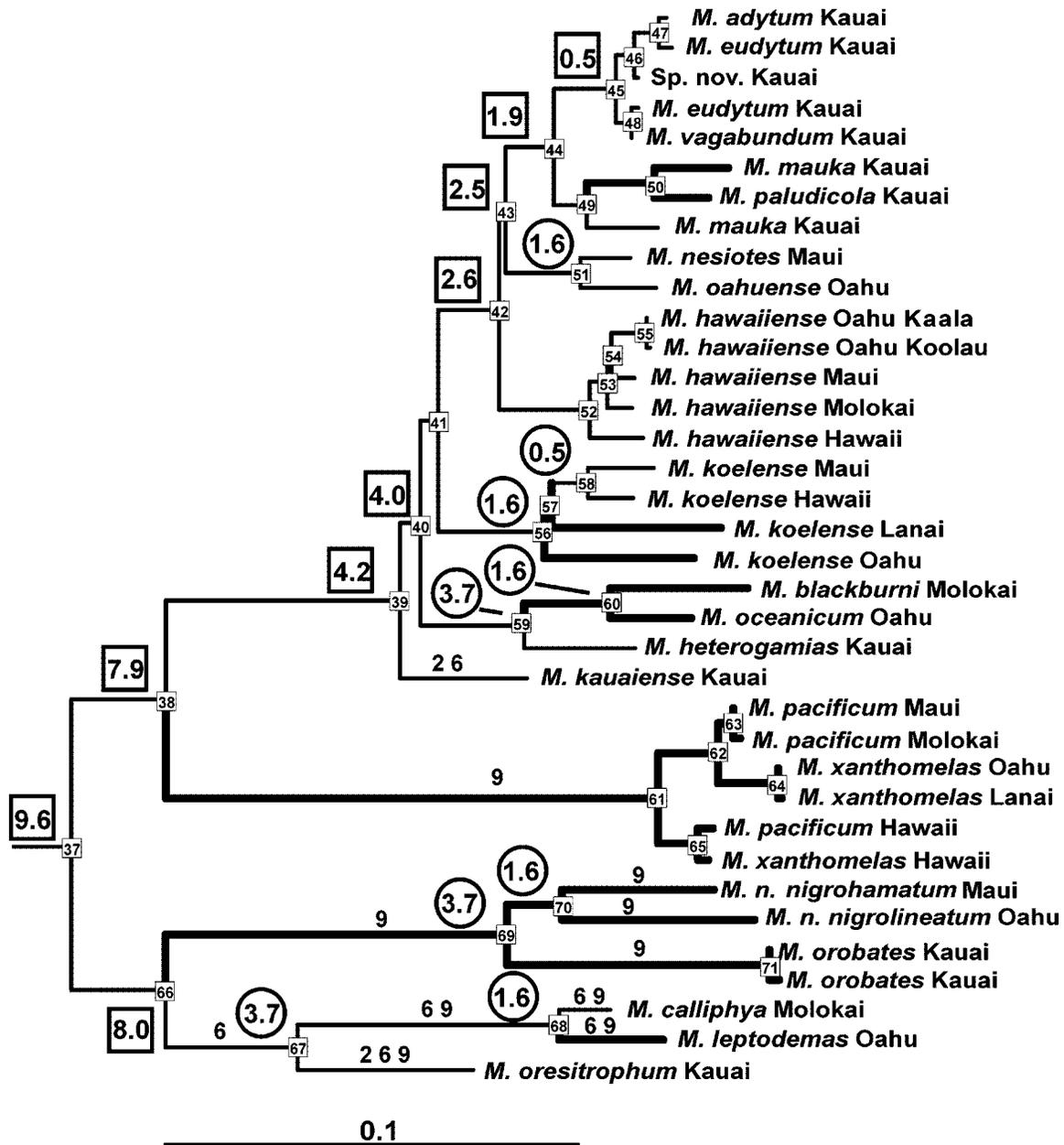


FIGURE 4. Unlocked ML mtDNA topology with dating information. For the dating analysis, branch lengths were calculated using the local clock of Yoder and Yang (2000) under four different rate scenarios. The first three scenarios allowed two possible rates and assigned the second rate to two, six, and nine branches, respectively. These branches are identified with a 2, 6, or 9. The fourth scenario allowed three possible rates, and branches marked with a 2 were assigned rate 1, branches shown in bold were assigned rate 2, all others were assigned rate 3. Nodes of the tree are numbered in accordance with Table 4. Nine nodes had their ages fixed following the suggestions of Fleischer et al. (1998). The ages assigned to these nodes (in millions of years, MY) are located in circles above and to the left of the node fixed. Local clock estimates for nodes of interest (MY) are in squares to the left of the node. Each estimate is the mean of the 36 calculated dates for that node.

#### Data Partition Congruence

The first SH test, performed with 34 specimens, parameters fixed, and 432 topologies compared, suggested that the mtDNA and EF-1 $\alpha$  data were in significant conflict (data not shown). The ML and MP mtDNA trees were significantly different from all 409 EF-1 $\alpha$  trees for all data sets tested. This test indicated that the large numbers of mtDNA and EF-1 $\alpha$  MP topologies were behaving

consistently under the SH test. We therefore cut the number of topologies from each of these sets to five and reran the SH test, this time with ML model parameters estimated. The results from this test are shown in Table 3. For the mtDNA data, all the mtDNA topologies and the combined nuclear/mtDNA topologies were good explanations of the data, but none of the EF-1 $\alpha$  topologies were. For the EF-1 $\alpha$  data, the EF-1 $\alpha$  topologies and the

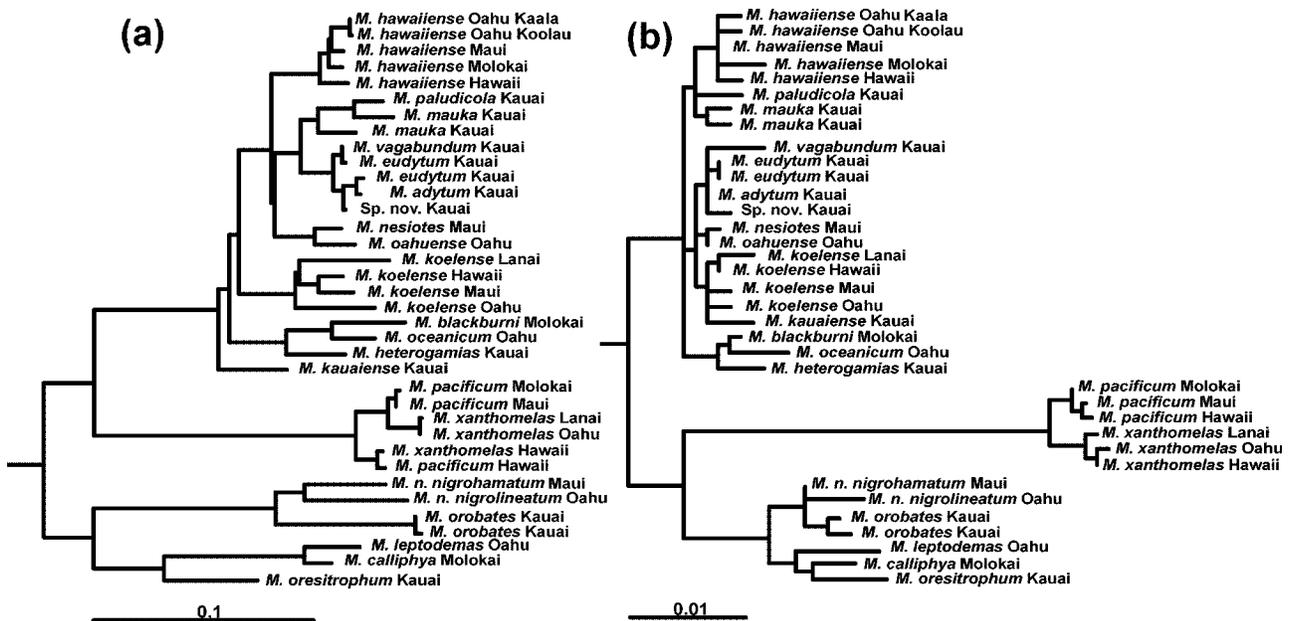


FIGURE 5. Phylograms of relationships of *Megalagrion* damselfly species. (a) Based on analysis of 1,287 bp of mtDNA sequence data. A full heuristic ML analysis was performed under the GTR+I+ $\Gamma$  model, with starting parameters estimated from an MP topology. This search was repeated with parameters reestimated from the ML topology. (b) Based on analysis of 1,039 bp of nuclear EF-1 $\alpha$  DNA sequence data. A search similar to that for the mtDNA data was conducted, but using a GTR+ $\Gamma$  model. For illustrative purposes, the location of the root is shown based on the constrained ML rooting analysis.

combined nuclear/mtDNA ML topology were good explanations, but none of the mtDNA topologies were. For the combined data set, all of the mtDNA topologies and the combined topologies were good explanations, but

none of the EF-1 $\alpha$  topologies were. The morphology tree of Polhemus (1997) did not adequately explain either molecular data set. These results indicate that the combined data ML topology is a significantly likely explanation of all data partitions and is part of the statistically indistinguishable group of best topologies for both loci.

TABLE 3. *P* values for the SH test for 15 trees and a variety of data partitions, testing the hypothesis that each tree is a significantly less likely explanation of the data than the best tree. Tested trees include the mtDNA ML tree, five mtDNA MP trees, the EF-1 $\alpha$  ML tree, five EF-1 $\alpha$  MP trees, the ML and MP trees from the combined data, and the morphology MP tree of Polhemus (1997).

Tested topology	Data set		
	Combined mtDNA	EF-1 $\alpha$	Combined mtDNA and EF-1 $\alpha$
mtDNA ML	<i>best</i>	0.026 <sup>a</sup>	0.709
mtDNA MP 1	0.842	0.026 <sup>a</sup>	0.647
mtDNA MP 2	0.875	0.026 <sup>a</sup>	0.662
mtDNA MP 3	0.948	0.026 <sup>a</sup>	0.745
mtDNA MP 4	0.960	0.020 <sup>a</sup>	0.749
mtDNA MP 5	0.808	0.026 <sup>a</sup>	0.657
EF-1 $\alpha$ ML	0.000 <sup>a</sup>	<i>best</i>	0.000 <sup>a</sup>
EF-1 $\alpha$ MP 1	0.000 <sup>a</sup>	0.893	0.000 <sup>a</sup>
EF-1 $\alpha$ MP 2	0.000 <sup>a</sup>	0.893	0.000 <sup>a</sup>
EF-1 $\alpha$ MP 3	0.000 <sup>a</sup>	0.702	0.000 <sup>a</sup>
EF-1 $\alpha$ MP 4	0.000 <sup>a</sup>	0.926	0.000 <sup>a</sup>
EF-1 $\alpha$ MP 5	0.000 <sup>a</sup>	0.861	0.000 <sup>a</sup>
Combined data ML	0.862 <sup>b</sup>	0.108 <sup>b</sup>	<i>best</i> <sup>b</sup>
Combined data MP	0.847	0.050 <sup>a</sup>	0.729
Morphology	0.000 <sup>a</sup>	0.009 <sup>a</sup>	0.000 <sup>a</sup>

<sup>a</sup>Topologies that scored significantly worse at the *P* = 0.05 level than the best score.

<sup>b</sup>These values indicate that the combined data ML topology is an adequate explanation of all data partitions taken individually, thus supporting the congruence of nuclear and mtDNA data sets.

#### Rooting and Phylogenetic Analysis

The ingroup topologies were rooted using the branch favored by the majority of bootstrap pseudoreplicates with the ingroup constrained and the outgroups free. This analysis resulted in two rooting hypotheses. The first, found with the mtDNA data, rooted the tree at the branch connecting the *M. orobates*/*M. oresitrophum* clade with the remainder of the taxa (clades are referred to by the name of the basal taxon; Fig. 5a). The second hypothesis, supported by the EF-1 $\alpha$  and combined data analyses, rooted the tree on the branch connecting the larger *M. orobates*/*M. oresitrophum*/*M. pacificum* clade with the remainder of the taxa (Figs. 3, 5b). Outgroup taxa formed a basal trichotomy in all analyses, offering no support to any hypothesis of sister status to the genus *Megalagrion* (Fig. 3, inset).

Selected models and estimated parameter values for ML analyses are listed in Table 2. LRTs and the AIC selected identical models in all cases. ML analysis of 36 ingroup individuals with the combined mtDNA resulted in a tree with a  $-\ln L$  of 7017.11 (Fig. 5a). Inspection of this phylogram and the corresponding bootstrap tree (Fig. 6) reveals that the mtDNA data were informative at both deep and tip nodes. Internal branches supporting some

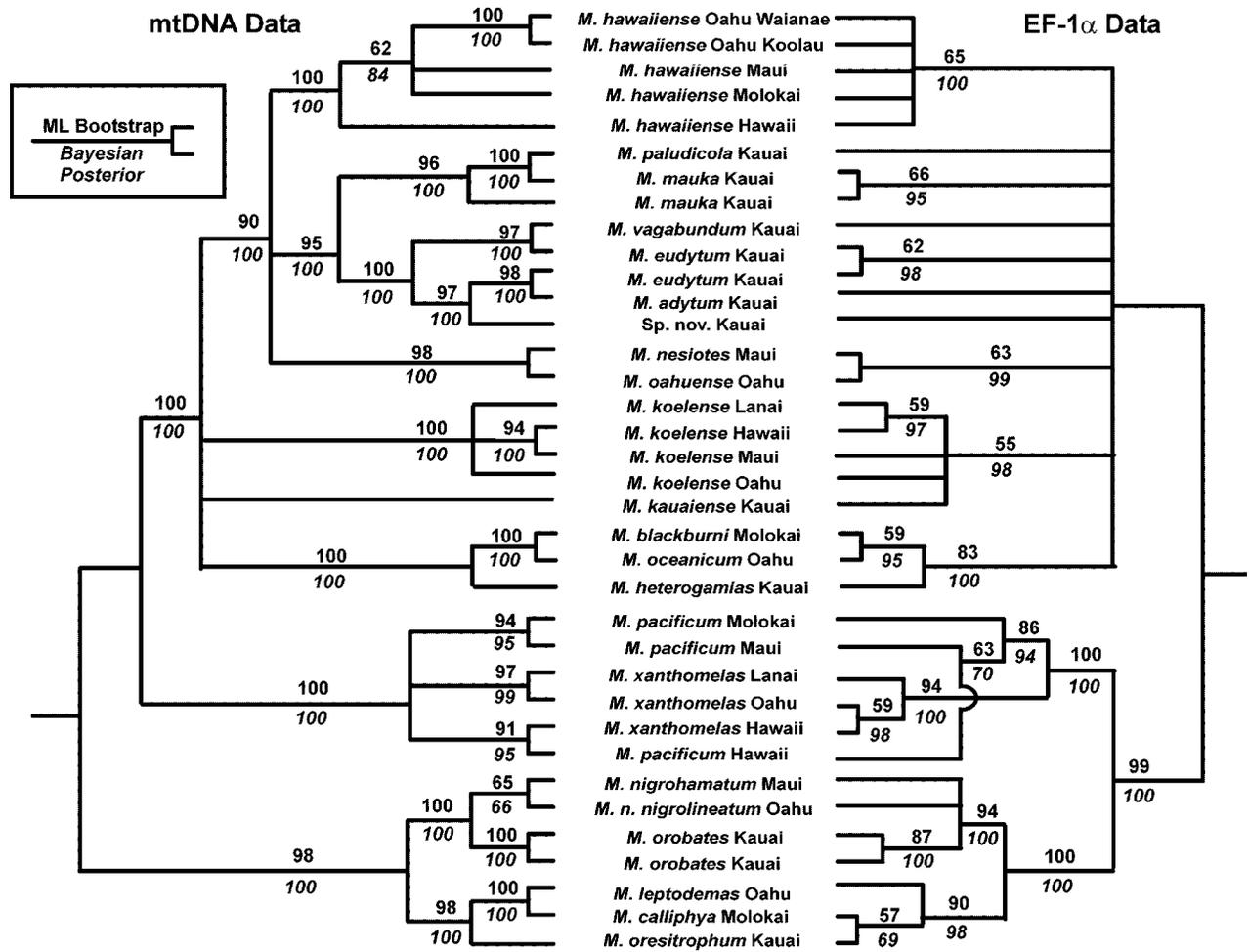


FIGURE 6. A comparison of the bootstrap and Bayesian hypotheses of *Megalagrion* damselfly species relationships based on two gene regions. Numbers above the line are ML bootstrap values based on 200 bootstrap pseudoreplicates. Numbers below the line are Bayesian posterior probabilities based on a 1 million-step MCMC simulation. A node is shown as resolved if its bootstrap value is  $\geq 55$  and its posterior probability is  $\geq 65$ . For illustrative purposes, the location of the root is shown based on the constrained ML rooting analysis.

midlevel nodes are short and disappear in the ML bootstrap and Bayesian trees, which are completely congruent for the mtDNA data.

ML analysis of the EF-1 $\alpha$  data resulted in a tree with a  $-\ln L$  of 2496.69. The EF-1 $\alpha$  ML phylogram (Fig. 5b) and bootstrap trees (Fig. 6) reveal a pattern similar to that produced by the mtDNA data, where most information supports deep and shallow splits and midlevel divergences again receive little support. An extremely long branch leads to the *M. pacificum* clade and may be responsible for the different mtDNA and nuclear rooting hypotheses. The ML bootstrap and Bayesian hypotheses for the EF-1 $\alpha$  data have some regions of incongruence, corresponding to short internal branches on the ML phylogram (Fig. 5b). In particular, several groups have extremely low bootstrap support (boxes in Fig. 7) but relatively high posterior probabilities.

The most likely tree resulting from the combined nuclear and mtDNA analysis has a  $-\ln L$  of 9829.75 (Fig. 3). This tree combines elements of both the mtDNA

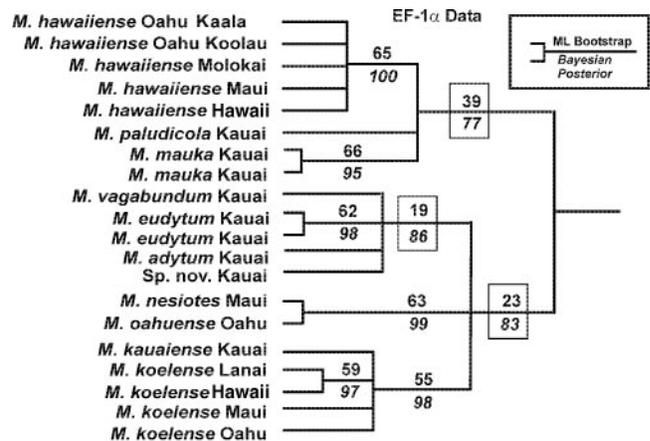


FIGURE 7. Species relationships of *Megalagrion* based on EF-1 $\alpha$  data. This figure is a portion of that shown in Fig. 6, but with three nodes added (boxed) that display a striking discordance between bootstrap proportions and posterior probabilities. See also Fig. 8.

TABLE 4. Estimated ages (MY) for all interior nodes of the mtDNA ML topology under four local clock branch length evolution scenarios. Ages were calculated nine separate times for each scenario of branch evolution, as the ages of nine separate nodes were fixed in turn. The range covered for each scenario reflects the different date estimates revealed by each successive node fixation. The mean and SD of the nine values for each scenario are noted. Node numbers correspond with those in Figure 4.

Node	Overall	Two rates											
		Nine branches			Two branches			Six branches			Three rates		
		Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
37	9.6	3.9–15.3	9.5	2.5	4.7–15.0	9.3	3.3	5.0–16.0	9.1	3.4	4.4–15.5	10.0	3.0
38	7.9	3.4–13.4	7.3	2.2	4.0–12.6	7.8	2.8	3.9–12.5	7.1	2.7	3.6–12.7	8.2	2.4
39	4.2	1.8–7.1	3.8	1.2	2.1–6.6	4.1	1.4	2.0–6.3	3.6	1.3	2.0–7.1	4.6	1.4
40	4.0	1.8–7.1	3.8	1.2	2.0–6.2	3.8	1.4	1.9–6.1	3.5	1.3	1.9–6.7	4.3	1.3
41	3.7	1.7–6.6	3.6	1.1	1.8–5.7	3.5	1.2	1.8–5.6	3.2	1.2	1.8–6.2	4.0	1.2
42	2.6	1.2–4.7	2.6	0.8	1.2–3.8	2.4	0.8	1.2–3.8	2.2	0.8	1.3–4.4	2.8	0.8
43	2.5	1.2–4.5	2.5	0.7	1.2–3.7	2.3	0.8	1.2–3.7	2.1	0.8	1.2–4.2	2.7	0.8
44	1.9	0.9–3.4	1.9	0.6	0.9–2.8	1.7	0.6	0.9–2.8	1.6	0.6	0.9–3.1	2.0	0.6
45	0.5	0.3–1.0	0.6	0.2	0.3–0.8	0.5	0.2	0.3–0.8	0.5	0.2	0.3–0.9	0.6	0.2
46	0.3	0.2–0.6	0.4	0.1	0.2–0.5	0.3	0.1	0.2–0.5	0.3	0.1	0.2–0.6	0.4	0.1
47	0.1	0.1–0.2	0.2	0.0	0.1–0.2	0.1	0.0	0.1–0.2	0.1	0.0	0.1–0.2	0.1	0.0
48	0.1	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0
49	1.5	0.7–2.9	1.6	0.5	0.7–2.3	1.4	0.5	0.7–2.3	1.3	0.5	0.7–2.4	1.5	0.5
50	0.8	0.4–1.6	0.9	0.3	0.4–1.3	0.8	0.3	0.4–1.3	0.7	0.3	0.4–1.2	0.8	0.2
51	1.1	0.5–2.0	1.1	0.3	0.5–1.6	1.0	0.3	0.5–1.6	0.9	0.3	0.5–1.9	1.2	0.4
52	0.9	0.4–1.6	0.9	0.3	0.4–1.3	0.8	0.3	0.4–1.3	0.7	0.3	0.4–1.5	1.0	0.3
53	0.5	0.3–1.0	0.6	0.2	0.2–0.8	0.5	0.2	0.3–0.8	0.4	0.2	0.3–0.9	0.6	0.2
54	0.5	0.2–0.9	0.5	0.1	0.2–0.7	0.4	0.2	0.2–0.7	0.4	0.1	0.2–0.9	0.5	0.2
55	0.0	0.0–0.1	0.1	0.0	0.0–0.0	0.0	0.0	0.0–0.0	0.0	0.0	0.0–0.1	0.0	0.0
56	2.1	1.0–4.0	2.2	0.7	1.0–3.3	2.1	0.7	1.0–3.3	1.9	0.7	0.9–3.2	2.1	0.6
57	2.0	1.0–3.7	2.0	0.6	1.0–3.1	1.9	0.7	1.0–3.1	1.8	0.7	0.9–3.0	1.9	0.6
58	1.1	0.5–1.9	1.1	0.3	0.5–1.6	1.0	0.3	0.5–1.6	0.9	0.3	0.5–1.8	1.1	0.3
59	2.5	1.2–4.5	2.5	0.8	1.2–3.8	2.4	0.8	1.2–3.8	2.2	0.8	1.1–3.7	2.4	0.7
60	1.5	0.7–2.9	1.6	0.5	0.8–2.4	1.5	0.5	0.8–2.4	1.4	0.5	0.6–2.2	1.4	0.4
61	1.2	0.6–2.4	1.3	0.4	0.6–1.9	1.1	0.4	0.6–1.9	1.1	0.4	0.5–1.6	1.0	0.3
62	0.6	0.3–1.2	0.7	0.2	0.3–0.9	0.6	0.2	0.3–0.9	0.5	0.2	0.2–0.8	0.5	0.2
63	0.1	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0
64	0.0	0.0–0.1	0.1	0.0	0.0–0.0	0.0	0.0	0.0–0.0	0.0	0.0	0.0–0.0	0.0	0.0
65	0.2	0.1–0.4	0.3	0.1	0.1–0.3	0.2	0.1	0.1–0.3	0.2	0.1	0.1–0.3	0.2	0.1
66	8.0	3.0–11.5	6.2	1.9	4.0–12.9	8.0	2.8	4.7–15.0	8.5	3.2	3.6–12.4	8.0	2.4
67	5.3	1.5–5.9	3.2	1.0	2.8–9.0	5.6	2.0	3.3–10.5	6.0	2.2	2.6–9.1	5.8	1.7
68	1.3	0.4–1.6	0.9	0.3	0.6–2.0	1.2	0.4	0.9–3.0	1.7	0.6	0.5–1.9	1.2	0.4
69	3.5	1.4–5.5	3.0	0.9	1.9–5.9	3.7	1.3	1.9–6.2	3.5	1.3	1.5–5.1	3.3	1.0
70	2.5	1.0–3.7	2.0	0.6	1.3–4.3	2.7	0.9	1.4–4.4	2.5	0.9	1.1–3.7	2.4	0.7
71	0.1	0.0–0.2	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0	0.0–0.1	0.1	0.0

topology and the EF-1 $\alpha$  topology and offers marginally better support to clades supported weakly by both (Fig. 3). For example, the EF-1 $\alpha$  data support the placement of *M. kauaiense* with the *M. koelense* clade, a relationship that has a bootstrap value (BP) of 57 and a posterior probability (PP) of 91 under the combined analysis. As for the mtDNA data, the combined data support the placement of *M. mauka* and *M. paludicola* with other Kauai species (BP-83; PP-100) rather than with *M. hawaiiense*, as the EF-1 $\alpha$  data weakly indicate (Fig. 5b). Neither data set can resolve the location of the *M. oahuense*/*M. nesiotese* clade, although their sister relationship is strongly supported by both sets, and by the combined data. The combined tree weakly supports the placement of the *M. heterogamias* clade with the *M. kauaiense* clade, a new insight not supported in either of the separate analyses.

#### Dating

Estimated dates for interior nodes using the local clock analysis of the mtDNA topology and data ranged from 0 to 9.6 MY (Table 4). Mean estimated dates for each node

varied little among the four models of branch length evolution, but much more within each model depending on which of the nine nodes was fixed in time. Means of all estimated dates for nodes of interest are plotted on the tree topology in Figure 4. These means reveal an estimated age for the first *Megalagrion* branching event of 9.6 MY. Two other nodes predate the formation of Kauai (5.1 MYA) by several million years. Polytomies correspond to dates of roughly 4.0 MY and 2.5 MY. A mini-radiation of four closely related Kauai species dates to 0.5 MY.

Dating analysis of the mtDNA topology under an alternative rooting hypothesis (suggested by the nuclear and combined data) resulted in very little change to the estimated dates. Of the 34 nodes shared by the two topologies, 28 showed slight increases in estimated age (up to 4.3% of the original value), and 6 showed slight decreases (up to 6.6%). Nodes with decreased age estimates were all in the *M. orobates*/*M. oresitrophum* clade. The estimated age for the first *Megalagrion* branching event under the alternative rooting was virtually unchanged at 9.5 MY.

## DISCUSSION

*Posterior Probabilities versus Bootstrap Proportions*

Our analysis revealed that Bayesian posterior probabilities, as expected, are not the functional equivalent of bootstrap proportions (Fig. 8). The posterior probability of a node is the probability of that clade given the

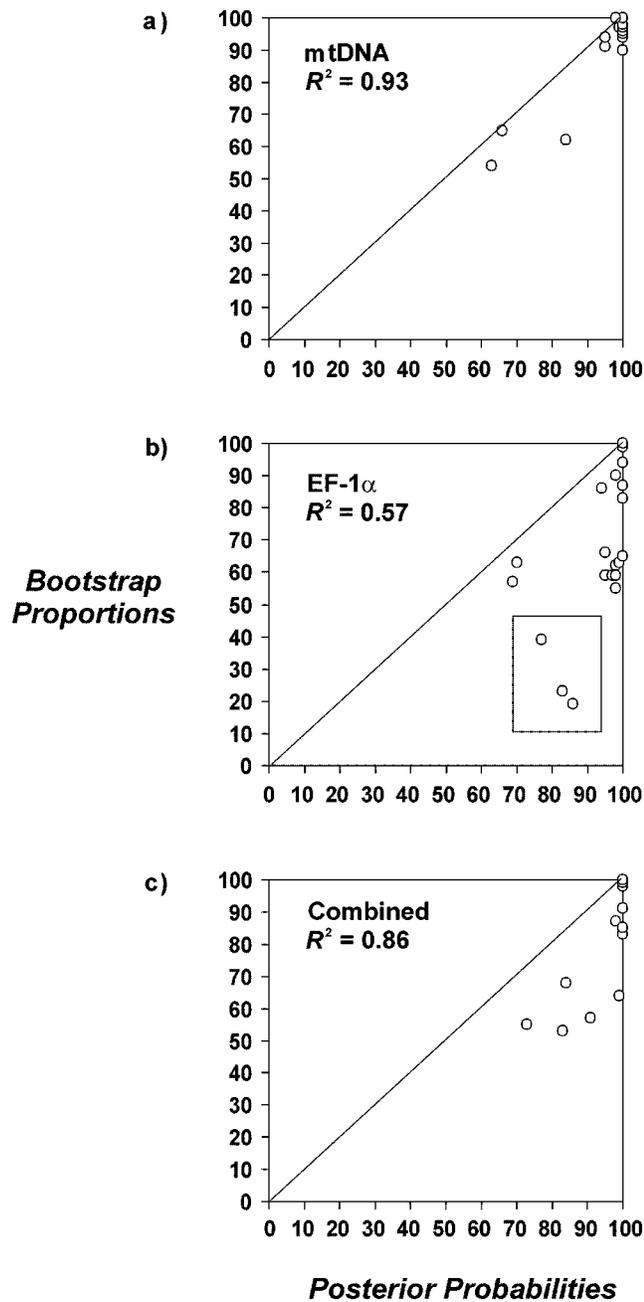


FIGURE 8. Comparisons of Bayesian posterior probabilities and ML bootstrap proportions from analysis of mtDNA data alone (a), nuclear data alone (b), and combined mtDNA and nuclear data (c). Diagonal lines represent unity. Pairs of values for each node were included only if  $BP \geq 55$  or  $PP \geq 65$ . Boxed data points in panel b correspond to nodes with notable discordance between the two measures, also indicated in Figure 7.

data and the model of evolution (see review by Lewis, 2001). Bayesian analysis produces a consensus tree containing nodes with the highest posterior probabilities. Bootstrapping is a nonparametric attempt to create a statistical distribution based on resampling the data set. Bootstrap proportions provide a biased measure of accuracy (underestimates when BPs are high and overestimates when BPs are low; Hillis and Bull, 1993). The bias varies depending on the number of taxa, the number of characters, and the location of internal branches. The two measures of nodal support are not meant to be equivalent, but they should be positively correlated apart from the extreme cases where sampling error plays a role.

In general, clade posterior probabilities are higher than the corresponding bootstrap proportions (Fig. 8). This difference is particularly acute for the EF-1 $\alpha$  analysis, perhaps because of the relative lack of signal in this data set. The values are sometimes in strong disagreement, as illustrated by three nodes on the EF-1 $\alpha$  bootstrap tree (Fig. 7). These nodes have extremely low bootstrap values (39, 19, and 23) but relatively high posterior probabilities (77, 86, and 83, respectively). Closer examination revealed that each of these nodes is supported by only one character, and the consistency index of that character is 1.0 in only the *M. hawaiiense* clade. As the bootstrapping procedure is carried out, these single sites are often absent from the pseudoreplicate data sets, and the nodes therefore collapse. The Bayesian analysis never subsamples the data set, and thus uses these characters in every topology it considers, giving them greater influence.

*Rooting, Outgroups, and the Origin of Megalagrion*

Of the four Pacific damselfly genera that are candidates as the sister taxon to *Megalagrion* (Polhemus, 1997; Polhemus et al., 2000), our primers were only successful in amplifying complete data sets from two: *Ischnura* and *Pseudagrion*. Our primer pairs failed on *Teinobasis palauensis* (Palau), and we obtained only mtDNA data from *Teinobasis rufithorax* (Papua New Guinea). We failed to obtain A6/A8 data and EF-1 $\alpha$  data from *Bedfordia* sp. from the Marquesas Islands. All primer pairs worked well on three species of *Pseudagrion* and two species of *Ischnura*, suggesting that perhaps these genera are more closely related to *Megalagrion*. The relationship of these coenagrionid genera to *Megalagrion* remains unknown. In all outgroup analyses, the outgroup taxa formed a basal trichotomy with *Megalagrion*, offering no support to any hypothesized sister relationship (Fig. 3 inset). Future studies of Pacific damselfly genera will need to include a molecule more suitable to observed divergences. Any search for the sister to *Megalagrion* should include North American taxa; our sampling of this fauna was incomplete. Furthermore, the challenge of Zimmerman (1948) to successfully examine *Pseudagrion* species from several localities remains to be adequately addressed.

*Damselfly Relationships*

Many authors have discussed the phylogenetic relationship of *Megalagrion* species (Perkins, 1899; Kennedy,

1929; Williams, 1936; Zimmerman, 1948; Polhemus, 1997). Several hypotheses are shared by these studies and were confirmed by the molecular data. First, we found *Megalagrion* to be monophyletic, and we identified many mitochondrial and nuclear nucleotide synapomorphies for the genus. Furthermore, all workers agreed that the large-bodied inhabitants of fast streams *M. heterogamias*, *M. oceanicum*, and *M. blackburni* are closely related. All workers also agreed on the existence of a clade containing *M. oresitrophum*, *M. leptodemias*, and *M. calliphya* that is closely related to *M. xanthomelas*, *M. pacificum*, *M. orobates*, and *M. nigrohamatum*.

There was less consensus of opinion among workers with respect to the affinities of other *Megalagrion* species. For example, Perkins (1899) and Zimmerman (1948) suggested that *M. adytum* and *M. eudytum* were closely allied, but Kennedy (1929) and Polhemus (1997), basing their analyses mainly on genitalia, considered these species to be distantly related. Molecular data show clearly that they are sister species (Fig. 3). These species are found in subtly different habitats, often with geographically overlapping or contiguous ranges. This pattern of morphological divergence in closely related sympatric species raises the intriguing possibility of divergent morphological selection to reduce hybridization.

Molecular data also offer insight into the traditionally difficult phylogenetic position of *M. hawaiiense*. Past workers proposed that *M. hawaiiense* was allied with (1) *M. vagabundum* (Perkins, 1899), (2) *M. adytum*, *M. jugorum*, and *M. nesiotetes* (Kennedy, 1929), (3) the *M. heterogamias* clade (Williams, 1936), (4) *M. eudytum*, *M. adytum*, *M. kauaiense*, *M. vagabundum*, and *M. williamsoni* (Zimmerman, 1948), or (5) only *M. eudytum* and *M. paludicola*, with a sister relationship to the *M. heterogamias* clade (Polhemus, 1997). Analysis with both molecular data sets positions *M. hawaiiense* in a large, unresolved clade (Figs. 5, 6), suggesting that a relatively rapid radiation led simultaneously to many of these species. Precise relationships of species in this clade, including *M. hawaiiense*, are difficult to understand because of a lack of accumulated support for any hypothesis.

The molecular data agree with the morphological hypothesis of Polhemus (1997) in several key ways. First, both support the sister status of *M. pacificum* and *M. xanthomelas* and the existence of a large clade containing the *M. oresitrophum*, *M. pacificum*, and *M. orobates* clades. Both also support the close relationship of the members of the *M. heterogamias* clade and the close alliance of *M. oahuense* with *M. nesiotetes*.

However, there are several areas of disagreement between the morphological and molecular data. First, molecular results suggest that the *M. heterogamias* clade is not closely related to the Kauai *M. mauka* clade, which is instead part of a larger clade of Kauai endemic species. Second, the best molecular tree is not fully resolved, whereas the Polhemus (1997) tree is, suggesting that periods of rapid evolution and species radiation led to much of the diversity within *Megalagrion* (Fig. 3).

Molecular analysis of a single male damselfly specimen from the Kanaele Swamp region of Kauai initially

identified as *M. kauaiense* resulted in a surprising placement on the tree. Closer inspection of this individual revealed that its terminalia were unlike any thus far known from *Megalagrion*, and it may represent a new species. Efforts should be undertaken to collect more individuals of this potentially undescribed taxon.

#### Hybridization

We have documented five potential cases of hybridization in *Megalagrion*. Individuals were identified to species based on morphology. First, the *M. pacificum* individual from Hawaii carried *M. pacificum* nuclear DNA but *M. xanthomelas* mtDNA (Jordan et al., 2001). Second, the *M. eudytum* individual from Kanaele Swamp, Kauai, bore *M. eudytum* nuclear DNA but *M. vagabundum* mtDNA. Third, one of the *M. orobates* carried both mtDNA and nuclear DNA that are identical to those of one of the *M. oresitrophum* specimens. This is particularly surprising given that these taxa show high genetic divergence at mtDNA loci (uncorrected = 0.11, GTR+I+ $\Gamma$  corrected = 0.18). Fourth, a single *M. nesiotetes* individual carried nuclear DNA that is identical to *M. oahuense* sequences, but mtDNA divergence between these species is about 0.03. Because these taxa occur on different islands and their sister status is supported by mitochondrial and nuclear data, this may only be a case of a shared ancestral nuclear allele rather than hybridization. The two *M. mauka* individuals carried similar EF-1 $\alpha$  sequences, but the individual from the Kalalau Trail carried mtDNA that is the sister to *M. paludicola* mtDNA.

#### Biogeography, Habitat Specialization, and Speciation

Two basic patterns of cladogenesis are common in Hawaii. Under the first, a product of the progression rule (Funk and Wagner, 1995), members of a clade disperse in tandem down the chain as new islands are created, in time forming assemblages of individual island endemics derived from disparate ancestors. This dispersal can be preceded by a rapid burst of evolution when a species initially reaches the islands and members of clades thus established may disperse to new islands together. This appears to be the case with *Orthotylus* mirids (Heteroptera), where discrete clades within the genus radiated onto particular host plant families in a rapid initial burst of adaptation on Kauai or an older island and have been tracking these respective hosts down the chain ever since (Polhemus, in press). *Blackburnia* carabid beetles also fall into this category (Liebherr and Zimmerman, 1998).

Under the second pattern, new islands are colonized by a single representative of a lineage already established in Hawaii that seeds a new burst of intransland cladogenesis. Adaptive bursts thus occur sequentially on each island in turn, but older island clades do not necessarily all disperse down the chain over time. This pattern is seen in Hawaiian crickets (Shaw, 1995) and in *Tetragnatha* (Gillespie et al., 1997) and giant *Orsonwelles* spiders (Hormiga et al., 2003).

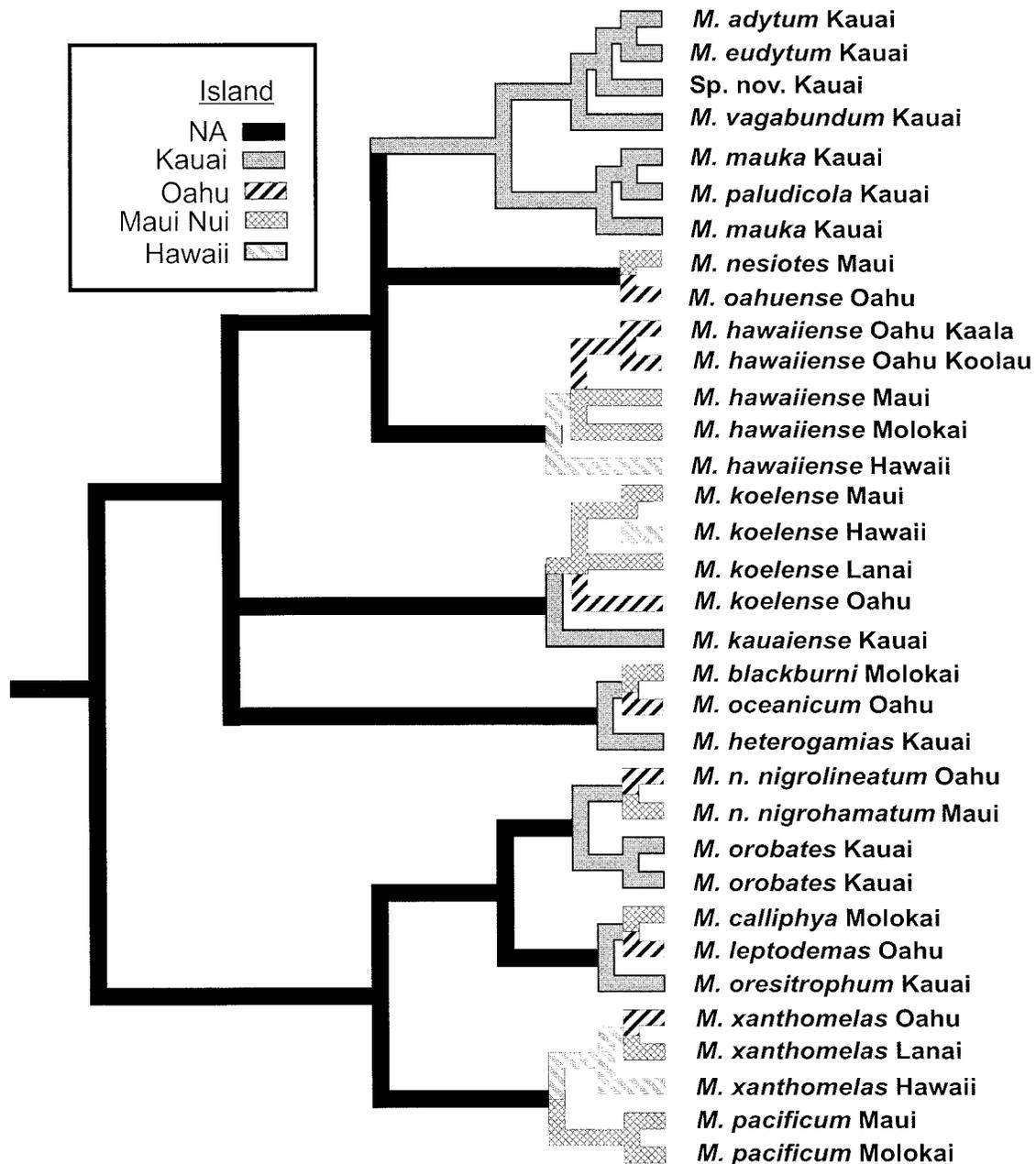


FIGURE 9. *Megalagrion* damselfly geographic distribution mapped onto the ML bootstrap topology for the combined data analysis.

Molecular analysis of *Megalagrion* diversification has revealed each of these patterns. The progression rule pattern is the most common, being seen in four clades (Fig. 9). The *M. kauaiense*/*M. koelense* clade is a particularly good example, with a ladderlike arrangement of taxa corresponding to movement down the island chain from Kauai to Hawaii (Figs. 3, 9). Other clades exhibiting patterns consistent with the progression rule include the *M. oresitrophum* clade, the *M. orobates* clade, and the *M. heterogamias* clade (Fig. 9). Within each of these clades, member species use the same larval habitat (Fig. 10b), and each clade has a species that is endemic to Kauai (Fig. 9). Habitat specificity in these clades likely evolved

on Kauai or an earlier island and was retained by new species as they arose on newly available islands.

*Megalagrion hawaiiense* displays an upchain pattern of radiation (Fig. 9). In this case, support is weak for the node separating Hawaii populations from those to the north (node 53 in Fig. 4; BP = 55, PP = 73 in Fig. 3). The local clock suggests a date of roughly 0.9 MY for the first branching event in this species, which is older than the island of Hawaii but well within the ages of Oahu and Maui Nui. If node 53 is collapsed, the origin of *M. hawaiiense* on Oahu or Maui Nui is not precluded.

These results are quite different from those seen by Losos et al. (1998), who found replicated miniradiations

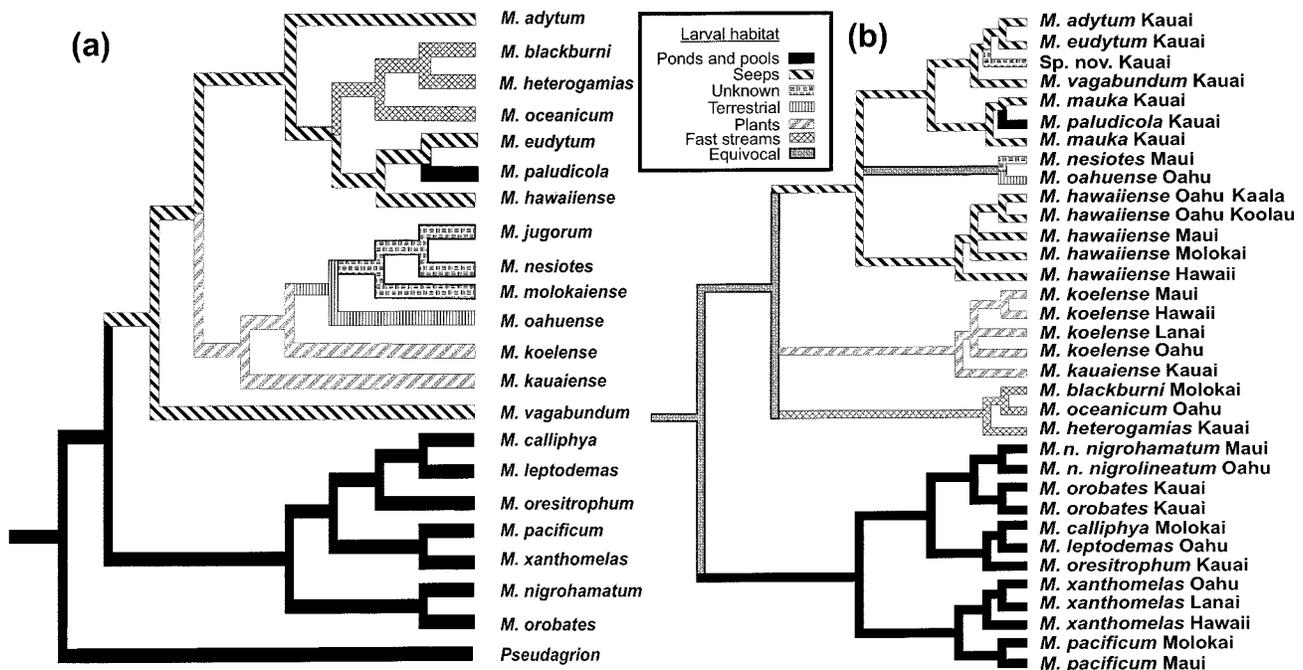


FIGURE 10. *Megalagrion* damselfly breeding habitat usage. (a) Mapped onto the morphological topology of Polhemus (1997). (b) Mapped onto the ML bootstrap topology for the combined data analysis.

of *Anolis* lizards on four islands of the Greater Antilles, and by Gillespie et al. (1997), who found essentially the same pattern in Hawaiian tetragnathid spiders. Each island contained the same set of habitat specialists, but species on each island were most closely related to one another. In contrast, each of the Hawaiian Islands contains a full complement of *Megalagrion* habitat specialists, but they are more closely related to members of their ecological guild than to their island mates. With one exception, speciation of *Megalagrion* has occurred between rather than within islands.

The single exception strongly supported by the molecular data is a clade found on Kauai consisting of *M. mauka*, *M. paludicola*, *M. vagabundum*, *M. eudytum*, *M. adytum*, and the potentially new species (Fig. 9). These species often exhibit overlapping ranges and have finely subdivided available habitats. For example, *M. paludicola* breeds in acidic upland pools that are markedly different from the lowland stream pools and ponds favored by *M. xanthomelas* and *M. pacificum*. Likewise, the seep habitats utilized by *M. adytum* and *M. eudytum* are not the same (Polhemus and Asquith, 1996). *Megalagrion eudytum* is found on wet vertical rock faces, generally in open situations adjacent to streams, whereas *M. adytum* prefers mossy rocks in damp, shaded gullies where water flow is basically seepage, with little or no surface flow. The estimated 1.9 MY age of this miniradiation within a 5-MY-old island seems to suggest that the high level of damselfly endemism on Kauai is due to relatively recent events. There may be a link to Pleistocene climatic change in this pattern. Kauai has the largest bogs of the Hawaiian chain, and many of its endemic damselfly species are

found in these acidic wetlands. The origin of these bogs is probably quite recent, but boggy refugia may have existed (J. Price, pers. comm.) as the Pleistocene climate experienced rapid variation (Hotchkiss et al., 2000).

The influence of ecology in *Megalagrion* speciation is illustrated by the fact that these damselflies breed in an astonishing array of habitats. Polhemus (1997) hypothesized that habitat usage in *Megalagrion* progressed from ancestral exploitation of pools and ponds to seeps and other semiwet habitats (Fig. 10a). From seeps, the evolutionary progression proceeded down two separate lines, the first to benthic regions of quickly flowing streams and the second to plant leaf axils and then damp fern leaf litter, a terrestrial habitat. Three species with unknown larval habitats formed a clade sister to the terrestrial species (Polhemus, 1997).

The molecular tree supports several aspects of Polhemus's analysis, including support for a clade of pond and pool dwellers as the sister to a clade containing all the other habitat types seen in *Megalagrion* and confirmation of the monophyly of several other groups whose members use identical habitats (Fig. 10b). Molecular data offer further support to a proposed sister relationship of *M. oahuense*, a known terrestrial breeder, and *M. nesioties*, which has been proposed to be terrestrial (Williams, 1936; Polhemus and Asquith, 1996; Polhemus, 1997).

However, molecular analysis offers a different hypothesis for the evolution of habitat usage (Fig. 10b). First, no convincing outgroup has emerged that allows polarization of the *Megalagrion* tree. All analyzed outgroups are quite distant from *Megalagrion*, and we have not identified a sister species or genus that would

allow us to infer the ancestral *Megalagrion* habitat. Furthermore, the molecular tree indicates no clear evolutionary progression in the exploitation of different habitats. Instead, there appears to have been two bursts of adaptation to novel habitats. The first, occurring roughly 4 MYA, led to the *M. kauaiense* clade (plant breeders), the *M. heterogamias* clade, (fast-stream breeders), and to another clade with species in diverse habitats. The second period of rapid radiation, occurring approximately 2.5 MYA, led to species that inhabit seeps, terrestrial habitats, and acidic upland pools (*M. paludicola*) and two species whose larvae and larval habitats are unknown.

#### *Adaptive Radiation and the Evolution of Island Endemics*

Hawaii's *Megalagrion* damselflies appear to satisfy Schluter's (1998:115) definition of adaptive radiation as the occurrence of "a burst of speciation and rapid phenotypic evolution under conditions of high ecological opportunity." Molecular dating indicates that bursts of *Megalagrion* speciation occurred on Kauai and Oahu. All lineages radiating from the first burst have member species on Kauai, which was the only island extant when this burst occurred 4 MYA. Thus, breeding in plants and fast streams probably evolved there at roughly the same time and moved down the chain as new islands emerged from the sea. Both Oahu and Kauai existed 2.5 MYA at the time of the second burst. However, two of the resultant lineages have representatives only on Oahu, which suggests that terrestrial breeding and *M. hawaiiense* originated there. A third radiation of species associated with bog habitats occurred rapidly about 1.9 MYA on Kauai, perhaps in response to newly emerging habitats associated with the bogs.

Members of this genus exhibit enormous diversity in habitat usage, which likely reflects high ecological opportunity at the time of radiation. Hawaii's streams lack entire orders of insects (e.g., Megaloptera, Ephemeroptera, Plecoptera, and Trichoptera) that dominate the continental benthos (Williams, 1936), which means that early *Megalagrion* had little competition in freshwater habitats. More striking, however, is the movement of *Megalagrion* lineages into the leaf axils of plants and terrestrial habitats beneath fern banks. The historical absence of ants and terrestrial mammals in Hawaii has likely facilitated this radiation into some of the most exceptional damselfly habitats in the world.

We propose a general model for the development of endemic island species in *Megalagrion*. First, there appears to be a minimum age necessary for an island to develop endemic damselfly species. This age is likely more than the 430,000 years of the island of Hawaii, which is the youngest island and has no endemic species. Three lines of evidence indicate that it may be <1.9 MY. First, *M. molokaiense* renders Molokai (1.9 MY) the youngest extant island with an endemic species. Second, Lanai and Maui also vie for status as the youngest islands with an endemic species, because although they share *M. jugorum*, they were connected as recently as 15,000 years ago. These two islands both formed about 1.3 MYA (Carson and Clague, 1995). Although *M. nesiotetes* occurs on

both Maui and Hawaii, the oldest volcano to host it is Haleakala of Maui, which is roughly 1.2 MY old. These examples can be taken to indicate that at least 430,000 years to 1.2 MY are required for endemic damselfly species to develop on Hawaiian islands. This estimate is supported by the dates of the two major *Megalagrion* radiations, which occurred roughly 4 MYA and 2.5 MYA, just over 1 MY after the estimated formation of Kauai (5.1 MYA) and Oahu (3.7 MYA).

Further reasoning along these lines offers insight into the shape of our estimated *Megalagrion* phylogenetic tree (Fig. 3). If roughly 1 MY are required for *Megalagrion* to speciate, this would put its arrival in Hawaii at about 11 MYA. Clague (1996) calculated that at least four islands over 1,000 m in elevation existed between 21 MYA and 5.1 MYA (the formation of Kauai). These islands (Laysan, Gardner, La Perouse, and Necker) are now eroded to atolls, shoals, pinnacles, and sea stacks, but all of them overlapped as high islands with our calculated dates for the existence of *Megalagrion* damselflies in the chain (Fig. 11). These islands would have offered suitable habitat for *Megalagrion* for many millions of years and likely hosted endemic single-island species. There are several lineages of *Megalagrion* that are characterized by extremely long basal branches that begin before the formation of Kauai (Fig. 4), including the clades of *M. oresitrophum*, *M. orobates*, *M. pacificum*, and the rest of the genus. All but the *M. pacificum* clade include many single-island endemics. These three clades date to branching events that occurred about 8 MYA. At that time, Laysan Island probably no longer had suitable damselfly habitat (Clague, 1996). If we assume that these clades were capable of producing single-island endemics on three islands presenting suitable habitat after 8 MYA (Gardener, La Perouse, and Necker), we estimate as many as nine *Megalagrion* species went extinct with the subsidence and erosion of these islands. If these extinct taxa used the same habitats as their extant sister species, we get a fascinating glimpse into the ecological characteristics of species that have been extinct for millions of years and have left no physical trace. Extinction of these endemic single-island species offers some explanation for the observed long branches.

The pattern observed on Kauai hints that more than nine species may have vanished with their host islands. A branching event leading to six endemic Kauai species occurred about 1.9 MYA (node 44, Fig. 4). This finding suggests that Kauai existed for over 3 MY before developing the bulk of its endemic damselfly species. If this kind of secondary radiation occurred on any of the three islands that hosted *Megalagrion* before Kauai, many more Hawaiian damselfly species may have become extinct. Furthermore, if any single island endemic species evolved between 9.6 and 8 MYA (e.g., on Laysan Island), they also have been lost.

The secondary radiation on Kauai is an important phenomenon that merits exploration. It occurred as the island reached a particular stage of senescence. There is undoubtedly some correlation between endemism and island age in Hawaii. This correlation may simply be

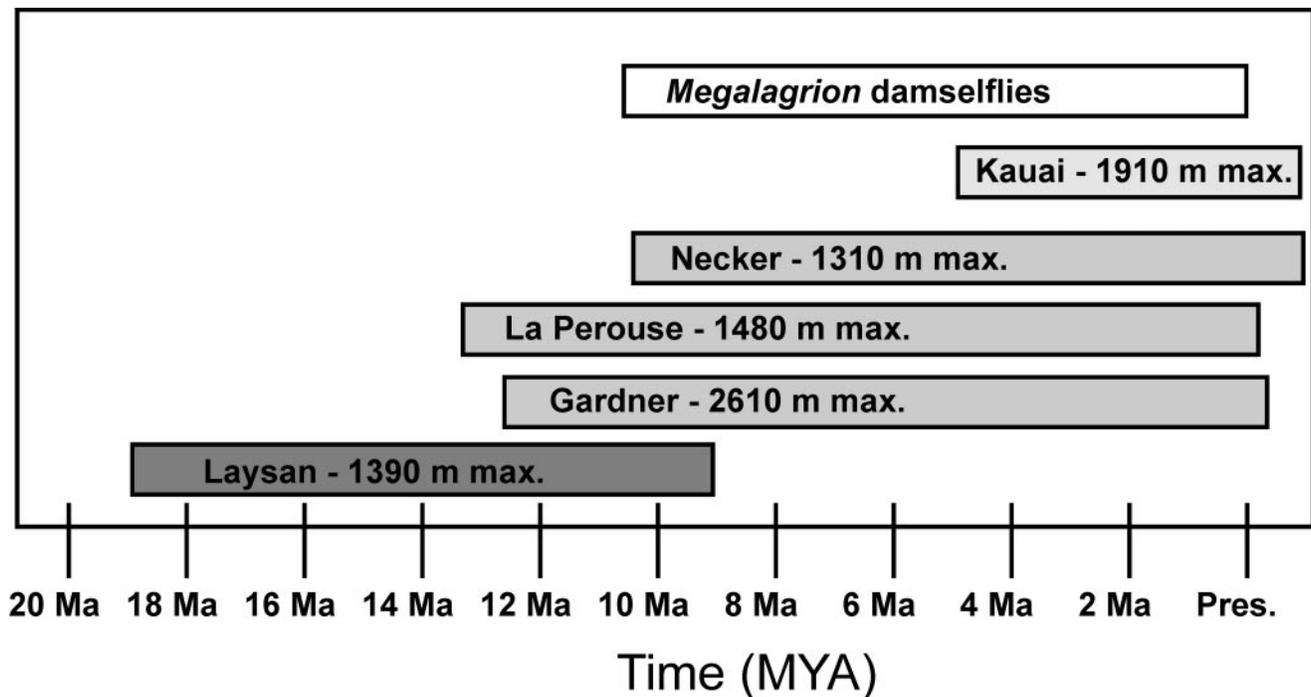


FIGURE 11. Timeline showing the overlap of *Megalagrion* occurrence and the existence of Kauai and four ancient Hawaiian high islands. Estimated maximum island elevations and dates of island formation and disappearance are from Clague (1996). *Megalagrion* dates were estimated using mtDNA data and the local clock procedure of Yoder and Yang (2000).

due to the evolutionary time required for speciation to occur, or age may also serve as a proxy for habitat diversification. These types of secondary radiations are probably not a predictable or uniform phenomenon. Oahu, Molokai, and Lanai are already past their major shield-building phases and now are deeply eroding, so that they will never develop elevated, boggy interior plateaus like that on Kauai and thus presumably cannot support secondary late stage *Megalagrion* radiations. The possibility of such radiations is still open on Maui and Hawaii, however. A combination of island age and habitat diversification is likely responsible for this intransland radiation, although this hypothesis remains untested. Fruitful research therefore remains to be done on the causes of the correlations between island age, habitat diversity, and arthropod speciation.

#### ACKNOWLEDGMENTS

Ron Englund provided specimens, field expertise, and insightful comments for this project, and we thank Paul Lewis for thoughtful discussion, assistance, and access to his Beowulf cluster. We are grateful to David Foote, Kent Holsinger, Mark McPeck, David Wagner, Ziheng Yang, and three anonymous reviewers for carefully reading the manuscript and to Jonathan Price and David Clague for helping us to understand Hawaii's geological and climatological development. Michael Whiting (Brigham Young University) kindly provided lab facilities and guidance for a portion of the initial work, and Karl Kjer (Rutgers University) served as a standard of quality and care in insect molecular systematics and, with Frank Carle, generously shared primer sequences. We thank Dave Swofford for advice and PAUP\*, Barbara Parsons for valuable assistance in the lab, Adam Asquith, Bill Puleloa, David Foote, David Preston, and Betsy Gagné for their help and support in the field, and Kristen Jordan for the loan of im-

portant specimens. We also thank our families for their support. This project was funded in part by the U.S. Geological Survey, the Canon National Parks Science Scholars Program, Sigma Xi, the Connecticut State Museum of Natural History Penner and Slater funds, the University of Connecticut Department of Ecology and Evolutionary Biology, the National Science Foundation Doctoral Dissertation Improvement program (grant 0073314 to S.J.), the NSF International Research Fellows Program (grant 0107373 to S.J.), and NSF grants to C.S. S.J. dedicates this paper to the memory of Danny Harris, good friend and systematist.

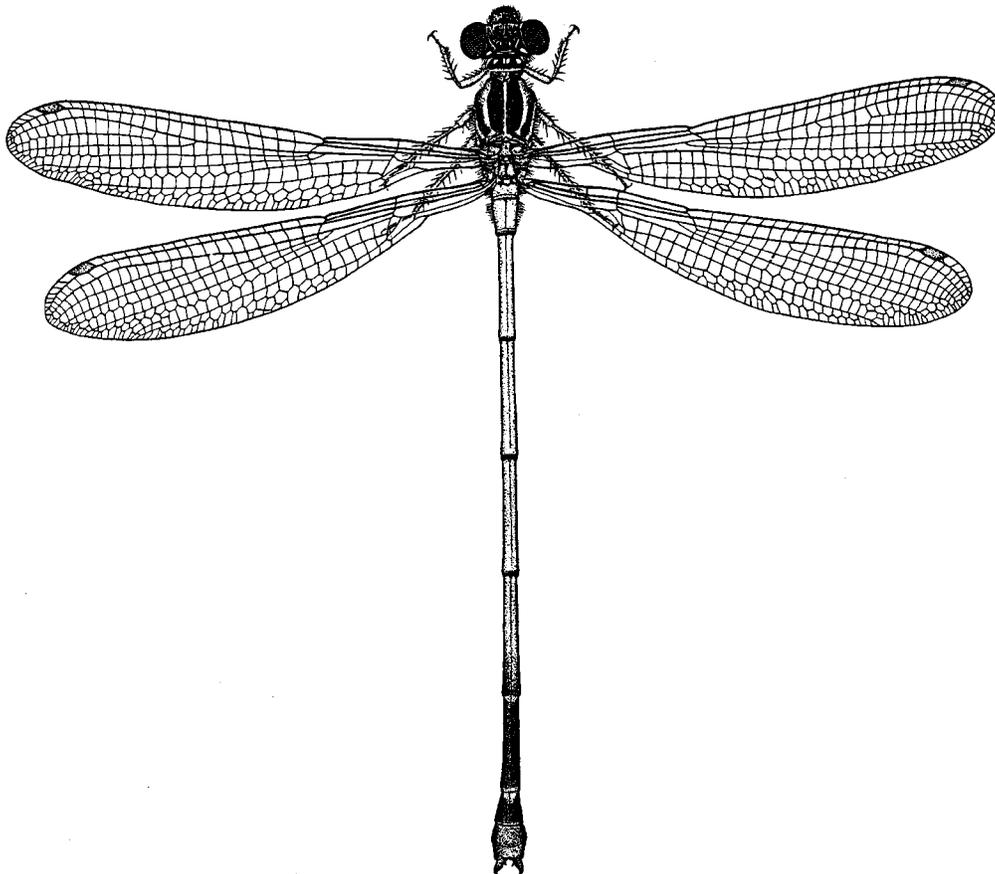
#### REFERENCES

- BARRACLOUGH, T. G., AND S. NEE. 2001. Phylogenetics and speciation. *Trends Ecol. Evol.* 16:391–399.
- BARRIER, M., R. H. ROBICHAUX, AND M. D. PURUGGANAN. 2001. Accelerated regulatory gene evolution in an adaptive radiation. *Proc. Natl. Acad. Sci. USA* 98:10208–10213.
- BUCKLEY, T. R., P. ARENSBURGER, C. SIMON, AND G. K. CHAMBERS. 2002. Combined data, Bayesian phylogenetics, and the origin of the New Zealand cicada genera. *Syst. Biol.* 51:4–18.
- BULL, J. J., J. P. HUELSENBECK, C. W. CUNNINGHAM, D. L. SWOFFORD, AND P. J. WADDELL. 1993. Partitioning and combining data in phylogenetic analysis. *Syst. Biol.* 42:384–397.
- CARLQUIST, S. 1980. Hawaii: A natural history: Geology, climate, native flora and fauna above the shoreline, 2nd edition. Pacific Tropical Botanical Garden, Lawai, Kauai.
- CARSON, H. L., AND D. A. CLAGUE. 1995. Geology and biogeography of the Hawaiian Islands. Pages 14–29 *in* Hawaiian biogeography: Evolution on a hot spot archipelago (W. L. Wagner and V. A. Funk, eds.). Smithsonian Institution Press, Washington, D.C.
- CHIPPINDALE, P. T., AND J. J. WIENS. 1994. Weighting, partitioning, and combining characters in phylogenetic analysis. *Syst. Biol.* 43:278–287.
- CHO, S., A. MITCHELL, J. C. REGIER, C. MITTER, R. W. POOLE, T. P. FRIEDLANDER, AND S. ZHAO. 1995. A highly conserved nuclear gene for low-level phylogenetics: Elongation factor-1-alpha recovers morphology-based tree for heliothine moths. *Mol. Biol. Evol.* 12:650–656.

- CLAGUE, D. A. 1996. The growth and subsidence of the Hawaiian-Emperor volcanic chain. Pages 35–50 in *The origin and evolution of Pacific Island biotas, New Guinea to eastern Polynesia: Patterns and processes* (A. Keast and S. E. Miller, eds.). SPB Academic, Amsterdam.
- CLAGUE, D. A., AND G. B. DALRYMPLE. 1987. The Hawaiian-Emperor volcanic chain. Pages 5–54 in *Volcanism in Hawaii*, U.S. Geological Survey professional paper 1350 (R. W. Decker, T. L. Wright, and P. H. Stauffer, eds.). U.S. Government Printing Office, Washington, D.C.
- CLARY, D. O., AND D. R. WOLSTENHOLME. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22:252–271.
- CRADDOCK, E. M. 2000. Speciation processes in the adaptive radiation of Hawaiian plants and animals. *Evol. Biol.* 31:1–53.
- DAIGLE, J. J. 1997. *Megalagrion mauka* spec. nov. from Kauai, Hawaii (Zygoptera: Coenagrionidae). *Odonatologica* 26:65–69.
- DEBRY, R. W., AND R. G. OLMSTEAD. 2000. A simulation study of reduced tree-search effort in bootstrap resampling analysis. *Syst. Biol.* 49:171–179.
- DONNELLY, T. W. 1990. The Fijian genus *Nesobasis*. Part 1: Species of Viti Levu, Ovalau, and Kadavu (Odonata: Coenagrionidae). *N.Z. J. Zool.* 17:87–117.
- FELSENSTEIN, J. 1983. Statistical inference of phylogenies. *J. R. Stat. Soc. A* 146:246–272.
- FLEISCHER, R. C., C. E. MCINTOSH, AND C. L. TARR. 1998. Evolution on a volcanic conveyor belt: Using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Mol. Ecol.* 7:533–545.
- FRATI, F., C. SIMON, J. SULLIVAN, AND D. L. SWOFFORD. 1997. Evolution of the mitochondrial cytochrome oxidase II gene in Collembola. *J. Mol. Evol.* 44:145–158.
- FUNK, V. A., AND W. L. WAGNER. 1995. Biogeographic patterns in the Hawaiian Islands. Pages 379–419 in *Hawaiian biogeography: Evolution on a hot spot archipelago* (W. L. Wagner and V. A. Funk, eds.). Smithsonian Institution Press, Washington, D.C.
- GILLESPIE, R. G., H. B. CROOM, AND G. L. HASTY. 1997. Phylogenetic relationships and adaptive shifts among major clades of *Tetragnatha* spiders (Araneae: Tetragnathidae) in Hawaii. *Pac. Sci.* 51:380–394.
- GILLESPIE, R. G., AND G. K. RODERICK. 2002. Arthropods on islands: Colonization, speciation, and conservation. *Annu. Rev. Entomol.* 47:595–632.
- GIVNISH, T., K. SYTSMAN, J. SMITH, AND W. HAHN. 1994. Thorn-like prickles and heterophylly in *Cyanea*: Adaptations to extinct avian browsers on Hawaii? *Proc. Natl. Acad. Sci. USA* 91:2810–2814.
- GOLDMAN, N., J. P. ANDERSON, AND A. G. RODRIGO. 2000. Likelihood-based tests of topologies in phylogenetics. *Syst. Biol.* 49:652–670.
- GRESSITT, J. L., AND C. J. DAVIS. 1969. Studies in the plagithmysines, endemic Hawaiian Cerambycidae (Coleopt.). *Proc. Hawaii. Entomol. Soc.* 20:331–393.
- HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 22:160–174.
- HILLIS, D. M., AND J. J. BULL. 1993. An empirical test of bootstrapping as a method from assessing confidence in phylogenetic analysis. *Syst. Biol.* 42:182–192.
- HOCH, H., AND F. G. HOWARTH. 1993. Evolutionary dynamics of behavioral divergence among populations of the Hawaiian cave-dwelling planthopper *Oliarus polyphemus* (Homoptera: Fulgoroidea: Cixiidae). *Pac. Sci.* 47:303–318.
- HORMIGA, G., M. ARNEDE, AND R. G. GILLESPIE. 2003. Speciation on a conveyor belt: Sequential colonization of the Hawaiian Islands by *Orsonwelles* spiders (Araneae, Linyphiidae). *Syst. Biol.* 52:70–88.
- HOTCHKISS, S., P. M. VITOUSEK, O. A. CHADWICK, AND J. PRICE. 2000. Climate cycles, geomorphological change, and the interpretation of soil and ecosystem development. *Ecosystems* 3:522–533.
- HOVEMANN, B., S. RICHTER, U. WALLDORF, AND C. CZIEPLUCH. 1988. Two genes encode related cytoplasmic elongation factors 1 alpha (EF-1 alpha) in *Drosophila melanogaster* with continuous and stage specific expression. *Nucleic Acids Res.* 16:3175–3194.
- HUELSENBECK, J. P., B. RANNALA, AND J. P. MASLY. 2000. Accommodating phylogenetic uncertainty in evolutionary studies. *Science* 288:2349–2350.
- HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
- JORDAN, S. 2001. Molecular systematics and phylogeography of Hawaii's *Megalagrion* damselflies. Ph.D. dissertation. University of Connecticut, Storrs, Connecticut.
- KANESHIRO, K. Y. 1974. Phylogenetic relationships of Hawaiian Drosophilidae based on morphology. Pages 102–110 in *Genetic mechanisms of speciation in insects* (M. J. D. White, ed.). Australia and New Zealand Book Co., Sydney.
- KENNEDY, C. H. 1917. Notes on the penes of damselflies (Odonata). 2. The close relations inter se of the Hawaiian agrionines. *Entomol. News* 28:9–14.
- KENNEDY, C. H. 1929. The origin of the Hawaiian odonate fauna and its evolution within the islands. *Proc. Fourth Int. Congr. Entomol.* 4:978–981.
- LARGET, B., AND D. SIMON. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Mol. Biol. Evol.* 16:750–759.
- LEWIS, P. A. 1998. Maximum likelihood as an alternative to parsimony for inferring phylogeny using nucleotide sequence data. Pages 132–163 in *Molecular systematics of plants II* (D. E. Soltis, P. S. Soltis, and J. J. Doyle, eds.). Kluwer Academic, Boston.
- LEWIS, P. O. 2001. Phylogenetic systematics turns over a new leaf. *Trends Ecol. Evol.* 16:30–37.
- LIEBHERR, J. K., AND E. C. ZIMMERMAN. 1998. Cladistic analysis, phylogeny and biogeography of the Hawaiian Platynini (Coleoptera: Carabidae). *Syst. Entomol.* 23:137–172.
- LIU, H., AND A. T. BECKENBACH. 1992. Evolution of the mitochondrial cytochrome oxidase II gene among ten orders of insects. *Mol. Phylogenet. Evol.* 1:41–52.
- LOSOS, J. B., T. R. JACKMAN, A. LARSON, K. DE QUEIROZ, AND L. RODRÍGUEZ-SCHETTINO. 1998. Contingency and determinism in replicated adaptive radiations of island lizards. *Science* 279:2115–2118.
- LUDWIG, K. R., B. J. SZABO, J. G. MOORE, AND K. R. SIMMONS. 1991. Crustal subsidence rates off Hawaii determined from  $^{234}\text{U}/^{238}\text{U}$  ages of drowned coral reefs. *Geology* 19:171–174.
- MCLACHLAN, R. 1883. Neuroptera of the Hawaiian Islands. Part I. Pseudo-Neuroptera. *Ann. Mag. Nat. Hist.* (V) 12:226–240.
- PAXINOS, E. E., H. F. JAMES, S. L. OLSON, M. D. SORENSON, J. JACKSON, AND R. C. FLEISCHER. 2002. MtDNA from fossils reveals a radiation of Hawaiian geese recently derived from the Canada goose (*Branta canadensis*). *Proc. Natl. Acad. Sci. USA* 99:1399–1404.
- PERKINS, R. C. L. 1899. Neuroptera. Pages 63–77 in *Fauna Hawaiiensis* (D. Sharp, ed.). Cambridge Univ. Press, Cambridge, U.K.
- PERKINS, R. C. L. 1910. Supplement to Neuroptera. Pages 693–696 in *Fauna Hawaiiensis* (D. Sharp, ed.). Cambridge Univ. Press, Cambridge, U.K.
- PERKINS, R. C. L. 1913. Review of the Neuroptera. Pages clxxv–clxxx in *Introduction to Fauna Hawaiiensis* (D. Sharp, ed.) Cambridge Univ. Press, Cambridge, U.K.
- POLHEMUS, D. A. 1997. Phylogenetic analysis of the Hawaiian damselfly genus *Megalagrion* (Odonata: Coenagrionidae): Implications for biogeography, ecology, and conservation biology. *Pac. Sci.* 51:395–412.
- POLHEMUS, D. A. In press. An initial review of the genus *Orthotylus* (Heteroptera: Miridae) in the Hawaiian Islands, with descriptions of twenty-two new species. *J. N.Y. Entomol. Soc.*
- POLHEMUS, D. A., AND A. ASQUITH. 1996. Hawaiian damselflies: A field identification guide. Bishop Museum Press, Honolulu.
- POLHEMUS, D. A., A. ASQUITH, AND S. MILLER. 2000. A new species of *Ischnura* from Rota (Odonata: Coenagrionidae), and a discussion of zygopteran zoogeography in the insular tropical Pacific. *Bishop Mus. Occas. Pap.* 62:5–12.
- PRICE, J., AND D. A. CLAGUE. 2002. How old is the Hawaiian biota? Geology and phylogeny suggest recent divergence. *Proc. R. Soc. Lond. B* 269:2429–2435.
- SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. *Molecular cloning: A laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- SCHLUTER, D. 1998. Ecological causes of speciation. Pages 114–129 in *Endless forms: Species and speciation* (D. Howard and S. Berlocher, eds.). Oxford Univ. Press, Oxford, U.K.

- SCHLUTER, D. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford, U.K.
- SELYS-LONGCHAMPS, E. DE. 1876. Synopsis des Agrionines, 5me Legion. Agrion. Bull. Acad. R. Belg. (II) 41:1–282.
- SHAW, K. L. 1995. Biogeographic patterns of two independent Hawaiian cricket radiations (*Laupala* and *Prognathogryllus*). Pages 39–56 in Hawaiian biogeography: Evolution on a hot spot archipelago (W. L. Wagner and V. A. Funk, eds.). Smithsonian Institution Press, Washington, D.C.
- SHIMODAIRA, H., AND M. HASEGAWA. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16:1114–1116.
- SIMON, C. 1987. Hawaiian evolutionary biology: An introduction. Trends Ecol. Evol. 2:175–178.
- SIMON, C., F. FRATI, A. BECKENBACH, B. CRESPI, H. LIU, AND P. FLOOK. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87:651–701.
- SIMON, C. M., W. C. GAGNÉ, F. C. HOWARTH, AND F. J. RADOVSKY. 1984. Hawaii: A natural entomological laboratory. Bull. Entomol. Soc. Am. 30:8–17.
- SIMON, D., AND B. LARGET. 2000. Bayesian analysis in molecular biology and evolution (BAMBE), version 2.02 beta. Department of Mathematics and Computer Science, Duquesne Univ., Pittsburgh, Pennsylvania.
- SWOFFORD, D. L. 1998. PAUP\*: Phylogenetic analysis using parsimony (\*and other methods), version 4. Sinauer, Sunderland, Massachusetts.
- SWOFFORD, D. L., G. J. OLSEN, P. J. WADDELL, AND D. M. HILLIS. 1996. Phylogenetic inference. Pages 407–514 in Molecular systematics, 2nd edition. (D. M. Hillis, C. Moritz, and B. K. Mable, eds.). Sinauer, Sunderland, Massachusetts.
- TARR, C. L., AND R. C. FLEISCHER. 1995. Evolutionary relationships of the Hawaiian honeycreepers (Aves, Drepanidinae). Pages 147–159 in Hawaiian biogeography: Evolution on a hot spot archipelago (W. L. Wagner and V. A. Funk, eds.). Smithsonian Institution Press, Washington, D.C.
- THACKER, R. W., AND M. G. HADFIELD. 2000. Mitochondrial phylogeny of extant Hawaiian tree snails (Achatinellinae). Mole. Phylogenet. Evol. 16:263–270.
- THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAC, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876–4882.
- WAGNER, W. L., AND V. A. FUNK (eds.). 1995. Hawaiian biogeography: Evolution on a hot spot archipelago. Smithsonian Institution Press, Washington, D.C.
- WIENS, J. J. 1998. Combining data sets with different phylogenetic histories. Syst. Biol. 47:568–581.
- WILLIAMS, F. X. 1936. Biological studies in Hawaiian water-loving insects. Part I. Coleoptera or beetles. Part II. Odonata or dragonflies. Proc. Hawaii. Entomol. Soc. 9:235–349.
- YANG, Z. 2000. Phylogenetic analysis by maximum likelihood (PAML), version 3.0. Univ. College, London, U.K.
- YODER, A. D., AND Z. YANG. 2000. Estimation of primate speciation dates using local molecular clocks. Mol. Biol. Evol. 17:1081–1090.
- ZIMMERMAN, E. C. 1948. Insects of Hawaii. Volume 2. Apterygota to Thysanoptera inclusive. Univ. Hawaii Press, Honolulu.
- ZIMMERMAN, E. C. 1978. Insects of Hawaii. Volume 9. Microlepidoptera (parts I and II). Univ. Hawaii Press, Honolulu.

First submitted 3 May 2002; reviews returned 23 July 2002;  
final acceptance 11 October 2002  
Acting Editor: Ziheng Yang



Adult male of the Oahu endemic *Megalagrion oceanicum*. Larvae of this large species (wingspan 58 mm) inhabit fast streams. Drawing from Williams (1936), original by J. Yamamoto.