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Diversification and evolution of Hawaiian *Megalagrion* damselflies (*Pinapinao*, Odonata: Coenagrionidae)

Ka Hoʻomāhuahua a me ke Kumu Hoʻomohala o nā Pinapinao Megalagrion o Hawaiʻi (Odonata: Coenagrionidae)

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Abstract

Hawaiʻi's pinapinao (*Megalagrion* McLachlan) comprises a radiation of 23 endemic damselfly species within Coenagrionidae. Despite being a unique study system for understanding geology's impacts on evolutionary processes among Odonata, the understanding of these damselflies' temporal, geographic and phylogenetic origins remains incomplete. Testing macroevolutionary hypotheses has been hampered by conflicting topologies. To resolve these uncertainties, we performed phylogenetic analyses including divergence time estimation with 90 nuclear loci (>50 kbp) and 2 mitochondrial loci (>1 kbp), sampling representatives from 37 genera within core Coenagrionidae and 90% of *Megalagrion* species, including multiple island populations. We used ancestral range estimations, diversification analyses, agent-based simulation modelling and ancestral state reconstruction to infer the group's origin and biogeography and assess traits' roles in diversification. Our findings indicate *Megalagrion*'s ancestor diverged from core Coenagrionidae in the early Eocene (∼51 MA) and diversified in the early Miocene (∼19 MA), suggesting *Megalagrion*'s MRCA predates Kauaʻi's emergence by 7–21 MY. Diversification analyses suggest a low rate after *Megalagrion* diverged from Coenagrionidae followed by a sudden increase around 19 MA, and simulation modelling supports extinction playing a significant role. Extant *Megalagrion* diversity is largely explained by ecological diversification into at least five clades with distinct breeding habitats that likely evolved on Northwestern Hawaiian Islands that are now-sunken seamounts. Speciation continued as descendants dispersed to current Hawaiian Islands as islands emerged. Species breeding in seeps further diversified within the island of Kauaʻi. Our results highlight including geologic changes over time in evolutionary studies and increase understanding of diversification patterns, biogeography and adaptive radiation on islands.

KEYWORDS
adaptive radiation, ancestral state reconstruction, extinction, Hawaiʻi, island biogeography, phylogenetics, simulation modelling

INTRODUCTION

Islands and their endemic biota stand out as study systems for understanding the effect of geology on evolutionary processes (Losos & Ricklefs, 2009a). Indeed, insular radiations have been and continue to be paramount to our discovery and understanding of evolution (Borregaard et al., 2016; Darwin, 1859; Losos & Ricklefs, 2009b; Patiño et al., 2017; Wallace, 1892; Whittaker et al., 2017). Understanding the effects of geologic history on the diversification, adaptation and distribution of life persists as a central theme in biology and greatly benefits from methodological advances in phylogenetics and modelling, including agent-based simulation modelling (Abar et al., 2017; Railsback & Grimm, 2012), phylogenetic software (Bouckaert et al., 2019; Kalyaanamoorthy et al., 2017; Minh et al., 2020), diversification modelling (Morlon et al., 2016) and genome-wide targeted DNA sequencing (Breinholt et al., 2018; Lemmon et al., 2012).

As one of the most isolated geologic features in the world, the Hawaiian archipelago presents a natural experiment in evolution (Cowie & Holland, 2008; Simon, 1987). For 85 million years, a persistent, somewhat stationary hotspot in the mantle beneath the Pacific tectonic plate has generated over a hundred volcanic islands. These islands have undergone a dynamic lifecycle: emerging from the ocean floor, drifting with the movement of the Pacific plate, weathering and eroding over time and ultimately subsiding below sea level. This process has resulted in the formation of the 6000-km long Hawaiian-Emperor seamount chain (Figure 1) (Clague, 1996). The relatively rapid and extreme changes in the environment due to island uplift and subsidence required inhabitants to migrate, adapt, or go extinct (Whittaker et al., 2010). With over 3000 km of consistent isolation from any continental coast for over 80 million years, immigration to the islands of the Hawaiian-Emperor chain has been limited to rare dispersal events, and colonizing biota have been completely isolated from their mainland relatives since their arrival.

The majority of Hawaiian flora and fauna is endemic, descended from individual ancient colonization events followed by diversification, with few possible exceptions (Cowie & Holland, 2008; Gillespie et al., 1994; Givnish et al., 2009; Hembry et al., 2021; Roderick & Gillespie, 1998). These include the Hawaiian silverswords (Baldwin et al., 1991; Baldwin & Sanderson, 1998; Blonder et al., 2016; Landis et al., 2018), Hawaiian lobeliads (Givnish et al., 2009), Hawaiian mints (Lindqvist & Albert, 2002; Tomlin et al., 2024), Hawaiian honeycreepers (Johnson et al., 1989), *Drosophila* spp. (Bonacum et al., 2005; Kambysellis et al., 1995; Lapoint et al., 2011, 2013, 2014; O'Grady et al., 2011; Russo et al., 1995), land snails (Cowie, 1992, 1995; Holland & Cowie, 2009; Régnier et al., 2015; Rundell et al., 2004), sword-tail crickets (Otte, 1994), *Hyposmocoma* Butler moths (Haines et al., 2014; Rubinoff & Schmitz, 2010), *Philodoria* Walsingham micromoths (Johns et al., 2018) and *Megalagrion* McLachlan damselflies (Jordan et al., 2003; Polhemus, 1997; Polhemus & Asquith, 1996). These endemic lineages are diverse in terms of numbers of species, morphology, behaviour and ecology, even in comparison to similarly aged continental lineages.

Among these lineages, the two dragonfly and 23 damselfly species (Odonata) of Hawaiʻi, collectively called *pinao* in ʻOlelo Hawaiʻi, have a significant role as freshwater invertebrates and are culturally important in ceremony, religion, fishing and play (Kahaulelio, 2003; Lili'uokalani, 2021; Maly et al., 2003; Pukui, 2022; Pukui & Elbert, 1986). They also serve as critical biological indicators of freshwater ecosystem health since other aquatic insect groups abundant on continents have not colonized Hawaiʻi (i.e., Ephermeroptera, Plecoptera and Trichoptera) (Englund et al., 2007; Golfieri et al., 2016; Gómez-Tolosa et al., 2021; Lee et al., 2018). Three separate odonate colonizations have led to two endemic dragonfly species (e.g., *Anax strenuus* Hagen and *Nesogonia blackburni* (McLachlan)) and the endemic damselfly genus *Megalagrion* (McLachlan, 1883) known as *pinapinao*, or *pinao ʻula* by Indigenous Hawaiians.

The endemic *Megalagrion* damselflies of Hawaiʻi are a useful study system to investigate the effects of geology on evolution and diversification. The genus includes 23 described species (21 extant) across seven islands (Niʻihau, Kauaʻi, Oʻahu, Maui, Molokaʻi, Lānaʻi, Hawaiʻi). *Megalagrion* is among the most significant radiations of Odonata in the Pacific, rivalled only by the radiation including *Nesobasis* Selys, *Vanuatubasis* Ober & Staniczek and *Nikoulabasis* Ferguson, Marinov, Saxton, Rashni & Bybee from Fiji and Vanuatu (38 species in total) (Donnelly & Marinov, 2024; Ferguson et al., 2023; Paulson et al., 2024) and perhaps a largely multi-archipelago, undescribed radiation including *Ischnura* Charpentier, *Hivaagrion* Hämäläinen &

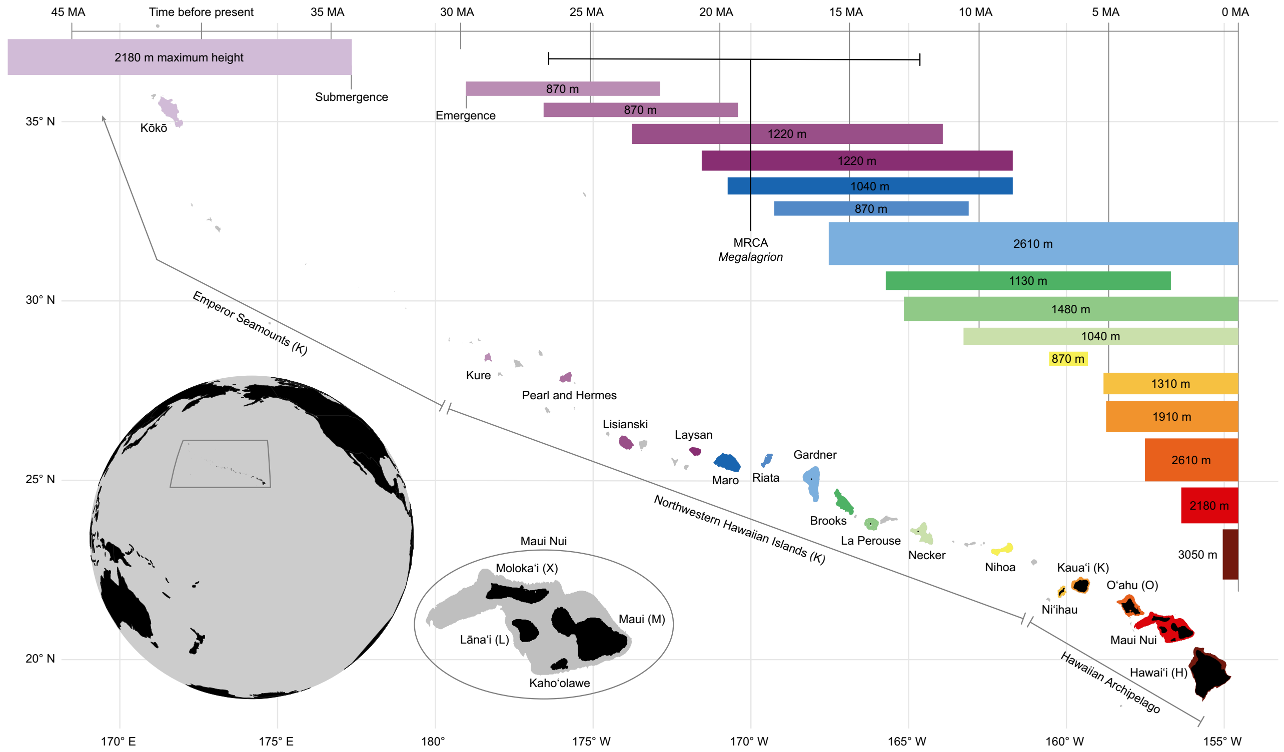

FIGURE 1 Grey and coloured islands represent contours 500 metres below sea level, which estimate the relative size and shape of past islands. Only significant islands are coloured, either due to their maximum estimated elevation above 1000 m, maximum estimated longevity of over 7 MY or geographic position as a potential stepping stone (Nihoa). No significant islands existed between 29 and 33 MA. No islands above 1300 m elevation existed between 16 and 33 MA. Darkly shaded portions of islands represent land that is currently above sea level (Hawai'i, Maui Nui, O'ahu, Kaua'i, Ni'ihau, Necker, La Perouse, Gardner). Coloured horizontal bars represent the time during which islands existed above sea level. The vertical width of each bar is proportional to the maximum estimated size of each island, given in metres. The estimated age of the most recent common ancestor of *Megalagrion* is marked with a vertical line and horizontal lines representing a 95% confidence interval.

Marinov, *Pacificagrion* Fraser and *Amorphostigma* Fraser (unpublished data). The unique ecological diversity of the Hawaiian damselflies is unlike that found in other Pacific groups. The 23 species of *Megalagrion* damselflies are found from sea level to above 1500 m and breed in a wide diversity of habitats, including lentic ponds and anchialine pools (i.e., with underground oceanic connections), lotic riffles, rheocrenes (streams flowing into channels), acidic upland bogs, phytotelmata (tank plants that hold water) and terrestrially in damp leaf litter (Polhemus & Asquith, 1996).

Members of *Megalagrion* diversified into at least five ecologically distinct lineages, constituting one of Hawai'i's best-known adaptive radiations (Jordan et al., 2003; Polhemus, 1997). This initial radiation was followed by speciation due to dispersal to new islands and largely follows the "progression rule" whereby ancestral lineages occupy older geographic areas and derived lineages occupy younger geographic areas (Hennig, 1966). Aside from a few exceptions on Kaua'i, *Megalagrion* species lack sister species on the same island, suggesting that within-island speciation could only have taken place on Kaua'i or an older island. Speciation on Kaua'i or older islands prior to the emergence of O'ahu (3.7 MA) is likely responsible for the majority of the ecological diversity, while more recent dispersal to newly formed islands (O'ahu, 3.7 MA, Maui Nui, 1.5 MA, Hawai'i, 0.5 MA) is likely responsible for the majority of species diversity (Figure 1) (Jordan et al., 2003).

Two previous phylogenetic studies hypothesized that adaptive radiation in *Megalagrion* occurred through ecological expansion into several new breeding habitats coupled with morphological expansion, including the evolution of two additional gill types. (Jordan et al., 2003; Polhemus, 1997). This includes novel "terrestrial" breeding behaviour such as in *M. oahuense*, which oviposits in and under banks of uluhe ferns, and *M. koelense*, which oviposits into the phytotelmata of 'ie'ie (*Freycinetia arborea*) (Polhemus & Asquith, 1996). Confirming the order and direction of these progressions relies on a sound understanding of phylogenetic relationships within *Megalagrion*. In addition, neither of the two previous phylogenetic analyses included a significant number of outgroups and were thus unable to confirm the phylogenetic position of *Megalagrion* within Coenagrionidae. Coenagrionid and odonate phylogenies with broader taxon sampling have recovered *Megalagrion* as sister to Ischnurinae (Dumont et al., 2010; Suvorov et al., 2021; Willink et al., 2024) and non-monophyletic within core Coenagrionidae O'Grady & May, 2003. Only O'Grady and May (2003) and Willink et al. (2024) included multiple *Megalagrion* samples. Thus, relationships within *Megalagrion* and its placement within core Coenagrionidae require clarification.

Furthermore, the temporal and geographic origins of *Megalagrion* are not well understood. Nearly 100 years ago, *Megalagrion* was hypothesized to have a South-East Asian origin based on its similarity to *Pseudagrion* (Kennedy), but with little evidence or explanation (Kennedy, 1929). Recent biogeographic analyses were inconclusive regarding the geographic origin of *Megalagrion* (Willink et al., 2024). The current estimates for the most recent common ancestor of *Megalagrion* vary widely (∼9.6–25 MA) (Jordan et al., 2003; Willink et al., 2024). Jordan et al. (2003) did not include fossil calibration points, and Willink et al. (2024) included fewer than 50% of *Megalagrion* species and no geographic calibration points. Analyses estimating the divergence time of *Megalagrion* from other coenagrionids (∼54 MA) also have limited taxon sampling of *Megalagrion* (Suvorov et al., 2021; Willink et al., 2024). The diversification of *Megalagrion* following its divergence from other coenagrionids approximately 54 MA and prior to the colonization of the current Hawaiian Islands hypothesized approximately 9.6–25 MA is not well understood. We hypothesize that extinction has played a significant role in the diversification of *Megalagrion* due to the age of the lineage and the variability of the ephemeral islands it has inhabited.

With the onset of targeted genome-wide sequencing, larger phylogenetic datasets are more accessible than ever before (Breinholt et al., 2018; Lemmon et al., 2012). Broader collaborations among odonate researchers and research including museum specimens have also increased the taxa available for sequencing (Abbott et al., 2022; Bybee et al., 2021; Goodman et al., 2023; Kalkman et al., 2022; Newton et al., 2023; Suvorov et al., 2021). We aim to clarify relationships within *Megalagrion* and test the phylogenetic position of *Megalagrion* within Coenagrionidae by building a dated phylogeny with extensive taxon sampling and increased molecular evidence. This phylogeny will allow us to infer temporal and geographic origins, reconstruct biogeographic and diversification dynamics and disentangle the contributions of extinction and adaptive radiation for the early diversification of *Megalagrion*.

To address these questions, we employed a multifaceted analytical approach that integrates phylogenetic, temporal, biogeographic and diversification analyses. We first reconstructed robust phylogenetic relationships using both concatenated maximum likelihood and coalescent-based methods (ASTRAL) to account for potential gene tree discordance from incomplete lineage sorting. We then estimated divergence times using fossil and geographic calibrations to establish a temporal framework for *Megalagrion* evolution. Within this phylogenetic and temporal context, we reconstructed ancestral ranges using probabilistic biogeographic models to infer colonization patterns across the Hawaiian archipelago. To test hypotheses about adaptive radiation, we performed ancestral state reconstruction of breeding habitat preferences and gill morphology—two key ecological and morphological axes along which Megalagrion has diversified. We complemented these approaches with diversification rate analysis (RPANDA) to identify lineages with significant rate shifts and agent-based simulation modelling to explore the role of extinction in shaping current diversity patterns. Together, these analyses provide a comprehensive framework for understanding how geological dynamics, ecological opportunity and extinction have shaped one of the Pacific's most remarkable odonate radiations.

METHODS

Taxon sampling

We gathered a total of 56 specimens from all 21 extant species of *Megalagrion*, plus one undescribed species (Table 1). Specimens were sourced from the personal collection of Steve Jordan—the Florida State Collection of Arthropods, Brigham Young University and fieldwork following local and state permitting requirements in Hawai'i. In addition to having complete sampling across the genus, we wanted to have representatives of each taxon from the different habitats in which they are found. With few exceptions, we obtained one representative of each species of *Megalagrion* from every major volcano, including multiple volcanoes on the same island, except the island of Hawai'i, where we obtained at least one specimen of each species on the island. We acknowledge the subspecies division between *M. nigrohamatum nigrohamatum* (Blackburn) and *M. nigrohamatum nigrolineatum* (Perkins) and include specimens from each subspecies. We did not obtain specimens from the two extinct species (*M. jugorum* (Perkins) and *M. molokaiense* (Perkins)) or from several extirpated populations of extant species. Historical dry specimens from both extinct species and several of these extirpated populations are present at the Bishop Museum in Honolulu, Hawai'i. However, DNA was not extracted from these specimens due to their taxonomic importance as irreplaceable types.

In addition to our sampling of *Megalagrion*, we sampled representatives from 37 of 58 valid genera across core Coenagrionidae (sensu Dijkstra et al., 2014), which included all genera previously hypothesized to be closely related to *Megalagrion* (Table S1) (Dumont et al., 2010; O'Grady & May, 2003). We attempted to sample all genera that may be closely related to *Megalagrion*. Of the 21 genera from core Coenagrionidae not included in our sampling scheme, 10 are monotypic and the other 11 genera account for a total of 55 species. Our purpose in including this level of outgroup sampling was to determine the placement of *Megalagrion* within Coenagrionidae and to help estimate molecular divergence times and ancient biogeography.

For diversification analyses with RPANDA, an extended taxon sampling set was used with additional species sampled from genera across core Coenagrionidae, totalling 411 samples (Table S2).

DNA extraction & sequencing

For each specimen, thoracic muscle tissue was extracted when possible; otherwise, a single leg was used. Specimens were vouchered at the Bean Life Science Museum, Brigham Young University in Provo, Utah. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504). For at least one specimen from

TABLE 1 *Megalagrion* taxon sampling.

Collection	Catalogue #	Species	Species author	Island	Locality	Collection date	Collector(s)
FSCA	GEODE7856	*M. mauka*	Daigle, 1997	Kauaʻi	Kalalau Tr.	13 Jul 1997	Daigle
Jordan Pers. Col.	OC2	*M. oceanicum*	McLachlan, 1883	Oʻahu	Kōloa Gulch	19 Jan 1995	Evenhuis, Polhemus, Preston
Jordan Pers. Col.	C3	*M. calliphya*	(McLachlan, 1883)	Molokaʻi	Waikolu	28 Aug 1997	Englund
Jordan Pers. Col.	NH6	*M. n. nigrohamatum*	(Blackburn, 1884)	Molokaʻi	Wailau Valley	18 Jul 1998	Englund, Jordan
Jordan Pers. Col.	L3	*M. leptodemas*	(Perkins, 1899)	Oʻahu	Maʻakua Str.	29 Jul 1998	Asquith, Englund, Jordan, Parsons, Simon
Jordan Pers. Col.	OB4	*M. orobates*	(Perkins, 1899)	Kauaʻi	Upper Moalepe Str.	10 Nov 1994	Polhemus
Jordan Pers. Col.	C48	*M. calliphya*	(McLachlan, 1883)	Hawaiʻi	N. branch Māʻili Str.	31 Dec 1998	Englund, Kanewa
Jordan Pers. Col.	NL6	*M. n. nigrolineatum*	(Blackburn, 1884)	Oʻahu	Waikāne Str., station 2	02 Jun 1995	Polhemus
Jordan Pers. Col.	X127	*M. xanthomelas*	(Selys, 1876)	Lānaʻi	Koele Lodge	26 Apr 1995	Polhemus, Preston
Jordan Pers. Col.	X123	*M. xanthomelas*	(Selys, 1876)	Molokaʻi	Wailau Stream	19 Jul 1998	Puleloa
Jordan Pers. Col.	X4	*M. xanthomelas*	(Selys, 1876)	Oʻahu	Tripler Army Med. Ctr	11 Sep 1997	Englund
Jordan Pers. Col.	KL9	*M. koelense*	(Blackburn, 1884)	Maui	Puʻu Kukui summit tr.	13 Aug 1997	Bartlett, Oppenheimer
Jordan Pers. Col.	KL12	*M. koelense*	(Blackburn, 1884)	Hawaiʻi	Olaʻa Puʻu Unit, HVNP	30 Jul 1996	Morris
Jordan Pers. Col.	KL24	*M. koelense*	(Blackburn, 1884)	Molokaʻi	Peʻepeʻōpae Bog Tr.	22 Aug 1991	Polhemus
Jordan Pers. Col.	PC21	*M. pacificum*	(McLachlan, 1883)	Hawaiʻi	N. branch Māʻili Str.	31 Dec 1998	Englund, Kanewa
Jordan Pers. Col.	PC31	*M. pacificum*	(McLachlan, 1883)	Maui	Haipuaʻena Str.	1998	Polhemus
Jordan Pers. Col.	PC7	*M. pacificum*	(McLachlan, 1883)	Molokaʻi	Wailau Valley	18 Jul 1998	Englund, Jordan
Jordan Pers. Col.	KA4	*M.* sp.		Kauaʻi	Kānaele Bog	22 Jul 1998	Asquith, Jordan
Jordan Pers. Col.	HW75	*M. hawaiiense*	McLachlan, 1883	Hawaiʻi	Honokane Nui Str.	02 Mar 1994	Polhemus
Jordan Pers. Col.	B34	*M. blackburni*	(McLachlan, 1883)	Maui	Haipuaʻena Str.	15 Jul 1998	Jordan
Jordan Pers. Col.	HW50	*M. hawaiiense*	(McLachlan, 1883)	Molokaʻi	Wailau Valley	18 Jul 1998	Englund, Jordan
Jordan Pers. Col.	HW61	*M. hawaiiense*	(McLachlan, 1883)	Hawaiʻi	Onomea Str.	25 Jul 1998	Jordan, Parsons, Simon
Jordan Pers. Col.	B1	*M. blackburni*	McLachlan, 1883	Molokaʻi	Waikolu	28 Aug 1997	Englund
Jordan Pers. Col.	M4	*M. mauka*	Daigle, 1997	Kauaʻi	Kalalau Tr.	23 Jul 1998	Jordan, Parsons
Jordan Pers. Col.	NS4	*M. nesiotes*	(Perkins, 1899)	Maui	E. Wailua Iki Str.	20 Jul 1994	Polhemus, Preston
Jordan Pers. Col.	KA3	*M. kauaiense*	(Perkins, 1899)	Kauaʻi	Mt. Kahili	10 Aug 1996	Polhemus
Jordan Pers. Col.	A1	*M. adytum*	(Perkins, 1899)	Kauaʻi	Waialae-Mōhihi Tr.	19 May 1995	Polhemus
BYU	OD2607	*M. hawaiiense*	(McLachlan, 1883)	Oʻahu	Kahawainui Gulch, Lāʻie Fls.	02 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
Jordan Pers. Col.	KL94	*M. koelense*	(Blackburn, 1884)	Lānaʻi	Mt. Hāʻalelepaʻakai	02 May 1993	Polhemus
Jordan Pers. Col.	KL43	*M. koelense*	(Blackburn, 1884)	Oʻahu	Mt. Kaʻala, Dupont Tr.	02 Aug 2000	Gagne, Jordan, Simon

(Continues)

TABLE 1 (Continued)

Collection	Catalogue #	Species	Species author	Island	Locality	Collection date	Collector(s)
BYU	OD2610	*M. n. nigrohamatum*	(Blackburn, 1884)	Maui	Kapalua Str.	05 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2611	*M. blackburni*	McLachlan, 1883	Maui	Olowalu Str.	06 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2612	*M. eudytum*	(Perkins, 1899)	Kauaʻi	Kalalau Tr., left of Hanakāpīʻai Fls.	13 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2613	*M. calliphya*	(McLachlan, 1883)	Maui	Kapalua Str.	05 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2614	*M. oahuense*	(Blackburn, 1884)	Oʻahu	Poamoho Tr.	03 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2615	*M. calliphya*	(McLachlan, 1883)	Maui	Kopiliʻula Str., above hwy.	08 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2616	*M. eudytum*	(Perkins, 1899)	Kauaʻi	Kalalau Tr., Hanakāpīʻai Str. below Fls.	13 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2617	*M. vagabundum*	(Perkins, 1899)	Kauaʻi	Kalalau Tr., Hanakoa Fls. Tr.	11 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2618	*M. oahuense*	(Blackburn, 1884)	Oʻahu	Poamoho Tr., Koʻolau Crest	03 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2619	*M. oresitrophum*	(Perkins, 1899)	Kauaʻi	Kauaʻikinanā Str., Mōhihi Rd.	16 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2620	*M. paludicola*	Maciolek & Howarth, 1979	Kauaʻi	Alakaʻi Swamp Tr.	15 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2621	*M. heterogamias*	(Perkins, 1899)	Kauaʻi	Alakaʻi Swamp Tr.	15 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2622	*M. heterogamias*	(Perkins, 1899)	Kauaʻi	Kalalau Tr., Waiahuakua Str.	10 Aug 2022	Bybee, Frandsen, Hadfield, Lin, Shuler
BYU	OD2678	*M. n. nigrohamatum*	(Blackburn, 1884)	Maui	ʻĪao Str.	04 Jul 1993	Daigle
Jordan Pers. Col.	C32	*M. calliphya*	(McLachlan, 1883)	Hawaiʻi	Olaʻa Puʻu Unit, HVNP	23 Jul 1996	Jordan
Jordan Pers. Col.	HW81	*M. hawaiiense*	(McLachlan, 1883)	Maui	Mt. ʻEke	23 May 1997	Jordan
Jordan Pers. Col.	E10	*M. eudytum*	(Perkins, 1899)	Kauaʻi	Kānaele Bog	22 Jul 1998	Jordan
Jordan Pers. Col.	W3	*M. williamsoni*	(Perkins, 1910)	Kauaʻi	Lumahai Valley	26–28 Nov 1998	Asquith
Jordan Pers. Col.	HW13	*M. hawaiiense*	(McLachlan, 1883)	Oʻahu	Mt. Kaʻala	14 Aug 1997	Preston, Butler
Jordan Pers. Col.	KL33	*M. koelense*	(Blackburn, 1884)	Oʻahu	Mt. Kaʻala, Dupont Tr.	20 Aug 2000	Jordan, Simon, Gagné

each genus, sequences of 1306 nuclear loci were obtained through anchored hybrid enrichment (AHE) sequencing using a probe set designed to capture single-copy orthologues of exons across odonate genomes (Bybee et al., 2021). For the remaining samples of *Megalagrion*, we captured a reduced set of 90 nuclear loci (>50 kbp) as well as two mitochondrial loci (~1000 bp). AHE sequencing was performed by Rapid Genomics (Gainesville, FL, USA) following their standard protocols. This genome-wide approach with multiple loci helps capture phylogenetic signals that represent the evolution of species as a whole rather than specific genes (Degnan & Rosenberg, 2006).

Sequence processing & quality control

AHE data were processed with a pipeline modelled after Breinholt et al. (2018). Raw sequence reads were cleaned, and adapters were removed using fastp v0.23.2 (Chen et al., 2018). The 90 nuclear and 2 mitochondrial loci were assembled following Goodman et al. (2023) using iterative baited assembly following Breinholt et al. (2018) with SPAdes genome assembler v3.15.4 (Bankevich et al., 2012) and the *Tanypteryx hageni* (Selys) reference genome (Tolman et al., 2023). A more closely related zygopteran or coenagrionid genome may have improved locus recovery and alignment accuracy. However, the anisopteran *Tanypteryx hageni* genome was used because probes were designed to be single copy with this genome and to be compatible with extended datasets across Odonata for further studies with broader taxon sampling. Sequences from the expanded probe set with 1306 nuclear loci were filtered to include only the 90 nuclear loci also present in the smaller probe set. Contamination filtering was performed by excluding identical sequences from different genera using the -ublast function of USEARCH v11.0.667 (Edgar, 2010). For each locus, only one orthologue was chosen for each taxon based on bit score, length and coverage. We excluded taxa for which fewer than 30% of the 92 loci (< 27 loci) were recovered. In our main sampling scheme, 1 of 104 samples (<1%) recovered fewer than 27 loci. In the extended sampling scheme used for RPANDA analyses, 28 of 411 samples (~7%) had fewer than 27 of the 92 loci. Some samples from both sampling schemes were sequenced with the expanded probe set of 1306 nuclear loci, all of which were filtered to include only the 90 nuclear loci present in the smaller probe set. AHE sequencing recovered not only the targeted loci but also some flanking regions on either side of each locus.

To align sequences for each locus, we used the L-INS-I algorithm from MAFFT v7.520 (Katoh & Standley, 2013). To ensure that the sequences aligned properly, we aligned the target region first, including the sequence used as a reference during assembly. The reference sequence was included to identify the position of the target region of the alignment. Then, we aligned the full-length sequences including the flanking regions to the alignment containing only the target region using the -addlong function of MAFFT. The target-region sequences (except for the target-region reference sequence) were then removed from the alignment, leaving an alignment of the full-length sequences, plus the target-region reference sequence. Aligned sequences were filtered using Aliscore 22.ii.2012 (Kück et al., 2010; Misof & Misof, 2009) and AliCUT v2.31 (Kück, 2009), which aim to remove alignment-ambiguous or randomly similar sites. We trimmed the ends of alignments until there was at least 50% taxon coverage.

This trimmed alignment was then split into three separate alignments—one for the target region (based on the position of the target-region reference sequence) and two for the flanking regions on each side. The two alignments for the flanking regions on either side of the target region were then concatenated. Loci with less than 50% taxon coverage were removed from further analysis. This process yielded two sets of alignments: one including only the target region and the other including only the flanking regions. The average pairwise distance between sequences in a concatenation of the flanking region alignments was more than three times the average pairwise distance between sequences in a concatenation of the target-region alignments, indicating that the target and flanking regions have significantly different rates of evolution. Therefore, we chose to partition the target and flanking regions separately in phylogenetic analyses.

Phylogenetic analysis

We performed our main phylogenetic analyses on two datasets. The first included a full alignment (both the target and flanking regions), and the second included a target-region-only alignment. We also performed phylogenetic analyses with the extended taxon sampling scheme and target-region-only alignment for RPANDA following the same procedure. For each analysis, we performed a partitioned maximum likelihood phylogenetic inference using IQ-TREE v2.2.0 (Minh et al., 2020). Our preliminary full partition scheme contained 92 or 184 partitions reflecting our alignments [92 loci × (1 target) or 92 loci × (1 target + 1 flanking)]. We used ModelFinder Plus (Kalyaanamoorthy et al., 2017) to select the optimal partition scheme by merging partitions until model fit ceased to improve and to find the best-fitting model of evolution for each partition according to the Bayesian Information Criterion (−m MFP + MERGE option). We employed the identified partition scheme and evolutionary models to infer a maximum likelihood tree. To assess branch support, we conducted 1000 ultrafast bootstrap replicates with nearest neighbour interchange optimization (−bnni 1000 option).

Coalescent species tree analysis

We inferred species relationships using a multispecies coalescent approach implemented in ASTRAL (Mirarab & Warnow, 2015; Zhang et al., 2018) via the ASTER package v1.16.3.4 (Zhang et al., 2023). Individual gene trees were first reconstructed for each of the 92 loci from the full alignments using IQ-TREE with ModelFinder Plus to select the best-fitting substitution model for each locus. ASTRAL estimates the species tree by finding the tree that maximizes the quartet score given the input gene trees, accounting for incomplete lineage sorting under the multispecies coalescent model. Branch support was assessed using local posterior probabilities, which represent the probability of a quartet given the gene trees. To quantify gene tree discordance, we calculated Robinson-Foulds (RF) distances between each gene tree and the species tree using the phangorn package v2.11.1 (Schliep, 2011) in R. To visualize gene tree discordance in *Megalagrion*, we pruned trees to include only *Megalagrion*, then used the DensiTree function from the phangorn package to display all gene trees simultaneously as a cloudogram and compared them to the species tree generated by ASTRAL.

Divergence time estimation

To generate a starting tree for Bayesian divergence time estimation in BEAST, we used our maximum likelihood tree generated from the target-region-only alignment. From this tree, we reconstructed a maximum likelihood fossil calibrated tree using the chronos function from ape v5.7.1 in R (Paradis et al., 2004). We used four fossils to calibrate the tree (*Agriocnemis lacteola* Selys, *Enallagma florissantella* Cockerell, *Diceratobasis worki* Poinar and *Ischnura velteni* Bechly, Table 2). These fossils were diagnosed using apomorphies in their respective descriptions, and none of them has been used as taxa in phylogenetic datasets previously, though *I. velteni* has been used for divergence time estimation (Blow et al., 2021). Each was placed at the node of the

TABLE 2 Fossil information.

Fossil taxon	Min age	Max age	Housed at	Museum number	Stratum	Locality	Age	Reference	Phylogenetic placement	Log-normal prior distribution
Agriocnemis lacteola	15.67	20.43	Shandong Provincial Museum, China		Shanwang Formation	Shanwang, Linqu County, Shandong Province, China	Miocene: Burdigalian (He et al., 2011)	Zhang, 1989	MRCA *Agriocnemis*	μ = 1.93 σ = 1.25 offset = 15.67
Ischnura velteni	13.65	20.43	Staatl. Museum für Naturkunde, Stuttgart, Germany	SMNS no. Do-5687	Dominican Amber	Dominican Republic, Unknown Mine	Miocene: Burdigalian (Nel et al., 2006)	Bechly, 2000	MRCA *Ischnura*	μ = 1.97 σ = 1.25 offset = 13.65
Diceratobasis worki	13.65	20.43	Private Collection of Jim Work, Ashland, Oregon, USA		Dominican Amber	Cordillera Septentrional, "La Toca" mine, Dominican Republic	Miocene: Burdigalian (Nel et al., 2006)	Poinar, 1996	Parent node *Diceratobasis*	μ = 1.97 σ = 1.25 offset = 13.65
Enallagma florissantella	33.9	37.2	Yale Peabody Museum, New Haven, CT, USA	YPM 26172	Florissant Formation	Florissant, Station 14, Colorado, USA	late Eocene: Chadronian (Evanoff et al., 2001)	Cockerell, 1908	MRCA *Enallagma*	μ = 1.53 σ = 1.25 offset = 33.9

most recent common ancestor of their respective genera. Because we only had one sample from *Diceratobasis* Kennedy, its most recent common ancestor node was the node connecting *Diceratobasis* and its sister. We constrained the age of the root using a wide range around previous estimates of the age of the core Coenagrionidae (70 ± 20 MA) (Suvorov et al., 2021). We used a strict clock substitution model (discrete substitution model with lambda smoothing parameter = 1).

We used the preliminary fossil calibration maximum likelihood tree from above as a starting tree for an unpartitioned Bayesian divergence time estimation using BEAST v2.7.6 (Bouckaert et al., 2014, 2019; Drummond & Rambaut, 2007). We used the maximum likelihood tree generated from an unpartitioned full alignment including both the target and flanking regions to constrain the topology using a multiple monophyletic constraint prior. We performed analyses with both fossil and geographic calibration (see below) priors, and with only fossil calibration priors (Table 3).

For both analyses, we used the birth–death diversification model, an uncorrelated relaxed clock substitution model, and a GTR + G + I model of evolution with all parameters estimated. We used a root age prior with a normal distribution around 70 MA with a standard deviation of 10 MA based on previous estimates of the age of the core Coenagrionidae (Suvorov et al., 2021). We used four fossil calibration points with log-normal prior distributions (Table 2, same as fossil calibrations for preliminary calibration). Log-normal fossil calibration prior distributions were offset so that the minimum was equal to the minimum age of the fossil, and μ was adjusted until the 95% quantile was near the previously estimated crown age for core Coenagrionidae (Suvorov et al., 2021). We ran the MCMC chain for at least 10,000,000 generations and saved a tree every 5000 generations.

For analyses with a geographic calibration, seven points were placed within *Megalagrion* clades with overwhelming phylogenetic evidence of following the progression rule, similar to previous studies (Haines et al., 2014; Johns et al., 2018). The emergence times of Oʻahu and Maui Nui were used for these calibration points. Within each clade, calibration points were placed at the most recent common ancestor of the Maui Nui/Hawaiʻi clade based on the emergence of Maui Nui and the most recent common ancestor of the Oʻahu + Maui Nui/Hawaiʻi clade based on the emergence of Oʻahu. The emergence time of Kauaʻi was not used for any geographic calibration points because taxa from Kauaʻi may have had ancestral ranges that once included islands that are now submerged. Geographic calibrations had a uniform prior distribution with a maximum equal to the emergence time of the island and a minimum of zero. Our geographic calibration strategy assumes that if a clade endemic to a younger range has a sister endemic to the next-oldest island, then the clade with the younger range does not predate the formation of its current range. Clades that do not have an extant sister on Kauaʻi are not included in this assumption, allowing for the possibility that the clade could have originated on Kauaʻi or older islands that are now submerged. For example, there is the possibility that the closely related terrestrially breeding species *M. oahuense* and *M. nesiotes* have an extinct sister that once inhabited Kauaʻi or an older island; therefore, this clade is not used to aid in geographic calibration.

TABLE 3 Divergence time estimation analyses.

Calibration	MRCA *Megalagrion*	MRCA *Megalagrion* + Ischnurinae	MRCA Ischnurinae	MRCA Agriocnemidinae + Ischnurinae	MRCA core Coenagrionidae	Generations
Fossil only	27.37 [17.93, 36.62]	57.91 [49.01, 66.70]	52.86 [45.58, 61.06]	65.41 [55.84, 76.30]	89.36 [71.58, 98.65]	16,720,000
Fossil + geographic	18.76 [12.21, 26.57]	51.34 [44.40, 58.43]	47.21 [41.52, 53.39]	57.86 [49.65, 67.20]	81.39 [64.58, 89.54]	107,220,000

The MCMC results were analysed for convergence and to determine the appropriate amount of burn-in using Tracer v1.7.2 (Rambaut et al., 2018). Maximum clade credibility trees were generated from the posterior trees using TreeAnnotator v2.7.6 with 20% burn-in. Node heights were set to common ancestor heights. To validate our calibration strategy and address potential concerns about interactions between multiple node age constraints, we performed prior-only analyses sampling from calibration priors without molecular data. We analysed all 16 key parameters including 12 calibration nodes (5 fossil-based, 7 geographic-based) and 4 evolutionary model parameters (birth rate, death rate, clock rate, tree height).

Ancestral range estimation

We performed ancestral range estimation using BioGeoBEARS v1.1.3 in R (Massana et al., 2015; Matzke, 2013a; Matzke, 2013b, 2014; Van Dam & Matzke, 2016). We use the dated tree from our BEAST analysis with both fossil and geographic calibration priors. Previous biogeographic analyses of Coenagrionidae with a higher-level taxonomic sampling, including *Megalagrion* and various Ischnurines, were not able to determine the geographic origin of *Megalagrion*. This is likely because *Megalagrion* is sister to the globally distributed diverse clade Ischnurinae, making it difficult to determine the origin of *Megalagrion* using only phylogenetic relationships and distribution data. We chose to focus our analysis on biogeographic patterns within *Megalagrion* rather than repeat an attempt to determine the origin of *Megalagrion*. We pruned the tree to include only *Megalagrion*. Operational taxonomic units in BioGeoBEARS analyses should be phylogenetically distinct, genetically isolated populations; therefore, we chose to preserve island-endemic populations of species present on multiple islands as separate operational taxonomic units. We collapsed multiple representatives of monophyletic island-endemic populations.

We coded islands into six areas (Figure 1). The first area (K) included Kauaʻi, Niʻihau and any unknown ancestral range, including islands that have since submerged. The second area (O) included Oʻahu. The third area (M) included Maui. The fourth area (L) included Lānaʻi. The fifth area (X) included Molokaʻi. The sixth area (H) included the island of Hawaiʻi. Kahoʻolawe was not included in the analysis because no *Megalagrion* damselflies have ever been recorded from the island. Area 'K' includes islands that have since submerged because some nodes in the tree are older than any of the existing main Hawaiian islands (Jordan et al., 2003). The islands of Maui Nui have been connected during the last glacial maximum 20–21 KA (Price & Elliott-Fisk, 2004). Oʻahu and Maui Nui have also been connected through Penguin Bank during periods of low sea level. Gene flow between *Megalagrion* populations on different islands of Maui Nui has been shown to be greater than between populations on Maui Nui and populations on the island of Hawaiʻi (Jones & Jordan, 2015; Jordan et al., 2005). We chose to code the islands of Maui Nui as separate areas because populations on different islands are still often phylogenetically distinct and have significant genetic differences, and marine channels represent a strong barrier to gene flow. By coding these islands separately, we also aimed to understand patterns of dispersal between the islands of Maui Nui and Oʻahu at a higher resolution.

We performed a time-stratified analysis, where areas and ranges were not allowed if none of the islands from that area had yet emerged (Table S3). The earliest estimated emergence times of islands were used to define available areas (D. Clague, 1996). Thus, area K was always available since it included submerged islands and Kauaʻi, which still exists. Area O was only available after the emergence of Oʻahu (3.7 MA). Areas M, L and X were only available after the emergence of the earliest land of Maui Nui (Penguin Bank, 2.2 MA). Area H was only available after the emergence of the first volcano of the island of Hawaiʻi (Kohala, 0.6 MA).

To estimate distance between islands, we drew contours at sea level around each area using the 2023 GEBCO Grid and terra v1.7.71 in R (GEBCO Compilation Group, 2023; Hijmans, 2024). We used the "distance" function from the terra R package to calculate the minimum distance between areas. These distances were used to create a distance matrix for DEC models with distance-dependent dispersal (+x). Dispersal multipliers were not applied, and the null range was not included (Massana et al., 2015).

Twelve separate models were run in BioGeoBEARS. These 12 models included the DEC, DIVALIKE and BAYAREALIKE base models, base models with jump dispersal (+J), base models with distance-dependent dispersal (+x) and base models with both jump dispersal and distance-dependent dispersal (+J + x). All 12 models were compared using the Akaike information criterion (AIC) (Table S3), and the model with the lowest AIC value was selected as our final result. We included the +J model in our analysis because jump dispersal leading to founder-event speciation is expected to have occurred in *Megalagrion* as individuals colonize new islands.

Diversification and extinction

RPANDA

We performed a diversification analysis using RPANDA v2.3 (Morlon et al., 2016) in R following a recent methodological development

(Mazet et al., 2023). We used a maximum likelihood tree with expanded taxon sampling which included approximately half of the described species for included genera. This tree was generated using a full-length alignment including both target and flanking regions. We used ape v5.7.1 in R to collapse monophyletic species with multiple samples. We dated the tree using TreePL v1.0 (Smith & O'Meara, 2012) with penalized likelihood. We applied the same four fossil calibration constraints as in our Bayesian divergence time estimation (Table 2). We ran TreePL first with the "prime" option to identify optimal smoothing and rate parameters and then ran TreePL with these parameters and the "thorough" option to ensure convergence. TreePL provides a computationally efficient alternative to Bayesian methods like BEAST, making it well suited for this dataset with extended taxon sampling. We listed all species of core Coenagrionidae according to the World Odonata List (Paulson et al., 2024). Sampling fractions were generated using RPANDA. A backbone tree was chosen to include 10 clades of interest across the tree, all containing at least 10 species (Table 4). Note that names for these clades are not meant to be valid taxonomic suggestions but are purely for convenience. All possible shift combinations were tested using five diversification models (BCST, BVAR, BCST_DCST, BVAR_DCST, BCST_DVAR). We did not test the BVAR_DVAR model which allows both birth and death rates to vary. The BVAR_DVAR model has been criticized for being problematic and producing unrealistic estimates, and it is not currently recommended for use with RPANDA (Burin et al., 2019; Mazet et al., 2023). We tested backbone option with "crown. shift" and "stem. shift" and set multi.backbone to "all". Model selection used AICc values, with the combination yielding the lowest AICc across all models considered optimal. We ran the shift estimates function with multi.backbone = "all" to explore parameter space. For the clades with estimated shifts, we calculated the difference between their diversification parameter values from the backbone (family-wide) diversification parameters to identify the magnitude and direction of rate changes in each shifted clade.

TABLE 4 RPANDA backbone clades.

Clade	Contains
Coenagrionidae	All
Core Coenagrionidae	All except Teinobasinae
Teinobasinae	*Teinobasis* + *Melanesobasis* + *Papuagrion*
Ischnurinae	Ischnurini, Nesobasini, Acanthagrionini
Pseudagrionini	*Pseudagrion* + *Caliagrion* + *Archibasis*
Pseudagrioninae	Pseudagrionini + *Neoerythromma*
Megalagrion	*Megalagrion*
Ischnurini	*Ischnura* + *Hivaagrion* + *Amorphostigma* + *Enallagma* + *Proischnura* + *Africallagma* + *Aciagrion* + *Coenagriocnemis* + *Amphiallagma* + *Azuragrion* + *Xiphiagrion*
Nesobasini	*Nesobasis* + *Nikoulabasis* + *Vanatubasis* + *Austrocnemis* + *Thaumatagrion*
Acanthagrionini	*Coenagrion* + *Amphiagrion* + *Anisagrion* + *Apanisagrion* + *Tuberculobasis* + *Denticulobasis* + *Telagrion* + *Leptobasis* + *Mesamphiagrion* + *Diceratobasis* + *Cyanallagma* + *Andinagrion* + *Homeoura* + *Acanthallagma* + *Acanthagrion* + *Aeolagrion* + *Oxyagrion*
Agriocnemini	*Agriocnemis* + *Mortonagrion*

Agent-based simulation modelling

Diversification analyses such as BAMM and RPANDA are useful tools for identifying potential patterns of speciation and extinction rates across clades and over time. However, neither of these analyses incorporates the dynamic geology of the Hawaiian Islands, which is an important driver of diversification patterns in *Megalagrion*, and has likely been a driver of diversification in *Megalagrion* for millions of years prior to the emergence of Kaua'i. Although we have no extant populations from these sunken islands, we simulated an island system like the Hawaiian Islands and allowed a simulated lineage to evolve in this island system for tens of millions of years. Using this simulation model, we explored potential diversification patterns in a lineage like *Megalagrion* and further tested our hypothesis that extinction played a significant role in the evolution of *Megalagrion* due to geologic change over time.

We created an agent-based simulation model in NetLogo v6.4.0 (Wilensky, 1999) following standard principles for designing agent-based models (ABMs) (Grimm & Railsback, 2012; Railsback & Grimm, 2012). The model is described following the Overview, Design concepts and Details (ODD) protocol (Supplementary methods) (Grimm, 2020; Grimm et al., 2006, 2010). The model is a generalized simulation of the evolution of a single lineage across an oceanic hotspot island system at a large time scale (10s of millions of years). The model can be divided into a geologic submodel and an evolutionary submodel. The geologic submodel models the creation, movement and disappearance of islands in an oceanic hotspot system. The simulated island system is modelled after patterns seen in the Hawaiian Islands, but is stochastic and generalized to simulate any oceanic hotspot system by adjusting geologic parameters. The evolutionary submodel models the evolution of a single lineage of organisms. This simulated lineage is modelled after *Megalagrion*, but is highly generalized and could be used to represent any lineage of organisms by adjusting biological and evolutionary parameters. Most biological and evolutionary parameters included in the model are not empirically known for *Megalagrion*, and/or do not correspond to easily measurable biological variables. Geologic, biological and evolutionary parameters of the model are set manually and held constant throughout each run. However, variables such as island size, population size, number of species, extinction events, island age, endemism, the preferred niche of a species, genetic diversity and others are not set, but are emergent from the output of the model.

To validate the geologic submodel, geologic parameters were set to values similar to those empirically found in the Hawaiian Islands. We ran 54 simulations of the model, each for 20 million years. For each run, we recorded the average length of the island chain, the average island size, average island age and average number of islands throughout the simulation. The average and standard deviation of these data across all simulations were used to compare simulated island chains to the Hawaiian Islands.

At the end of each of these 54 simulations, we recorded the total number of species ever created and the number of extant species. The average and standard deviation of these data across all simulations were used to calculate the average speciation rate, extinction rate and net diversification rate across all simulations.

Ancestral state reconstruction

We performed stochastic character mapping on our maximum likelihood tree generated from a full alignment including both target and flanking regions using the make.simmap function of the R package phytools v2.1.1 (Revell, 2024) with the empirical Bayes method. Prior to ancestral state reconstruction, we selected the best evolutionary transition rate model by fitting three models to the data using the fitMk function (equal rates, symmetric rates and all rates different) and evaluating model support based on AIC scores. We mapped breeding habitat and gill type separately. Characters were coded based on previous literature (Table 5) (Jordan et al., 2003; Polhemus, 1997; Polhemus & Asquith, 1996). Gills were coded as being foliate, lanceolate or saccate. Breeding habitats were coded as lentic, lotic, seep, phytotelmata or terrestrial. The breeding habitat and gill type of *M.* sp. (Jordan et al., 2003) and *M. mauka* Daigle are not known. Therefore, two separate analyses were performed. For the first analysis, the character states of these species were given a flat prior distribution. The character states for these species were then estimated the same way character states were estimated for internal nodes. For the second analysis, these taxa were removed. We performed 10,000 simulations and then summarized all simulations by measuring the proportion of simulations that recovered each state at each node.

TABLE 5 Character matrix.

Species	Gill type	Breeding habitat
M. adytum	Lanceolate	Seep
M. blackburni	Lanceolate	Lotic
M. calliphya	Foliate	Lentic
M. eudytum	Lanceolate	Seep
M. hawaiiense	Lanceolate	Seep
M. heterogamias	Lanceolate	Lotic
M. kauaiense	Saccate	Phytotelmata
M. koelense	Saccate	Phytotelmata
M. leptodemas	Foliate	Lentic
M. mauka	Lanceolate	Unknown
M. nesiotes	Saccate	Terrestrial
M. n. nigrohamatum	Foliate	Lotic
M. oahuense	Saccate	Terrestrial
M. oceanicum	Lanceolate	Lotic
M. oresitrophum	Foliate	Lentic
M. orobates	Foliate	Lentic
M. pacificum	Foliate	Lentic
M. paludicola	Lanceolate	Lentic
M. vagabundum	Saccate	Seep
M. williamsoni	Unknown	Unknown
M. xanthomelas	Foliate	Lotic
M.sp..	Unknown	Unknown

RESULTS

Phylogenetics

Placement of *Megalagrion* within Coenagrionidae

Our maximum likelihood tree with a full alignment including both target and flanking regions confirms that *Megalagrion* is monophyletic and sister to Ischnurinae with 100% ultrafast bootstrap support (Figure 2, see Supplementary Information, Figure S1). Our tree generated from a target-region-only alignment also agrees with 100% BS. This sister relationship is consistent with previous phylogenies with fewer molecular characters and/or decreased coenagrionid taxon sampling (Dumont et al., 2010; Suvorov et al., 2021; Willink et al., 2024). This placement of *Megalagrion* is only somewhat consistent with previous morphological phylogenetic analysis of Coenagrionidae, which included two polyphyletic *Megalagrion* samples (O'Grady & May, 2003).

Relationships within *Megalagrion*

Results are shown in Figure 3.

Eight subclades were recovered within *Megalagrion* (Figure 3):

1. *M. xanthomelas* (Selys) + *M. pacificum* (McLachlan)
2. *M. orobates* (Perkins) + *M. nigrohamatum* (Blackburn)
3. *M. oresitrophum* (Perkins) + *M. leptodemas* (Perkins) + *M. calliphya* (McLachlan)
4. *M. heterogamias* (Perkins) + *M. oceanicum* McLachlan + *M. blackburni* McLachlan
5. *M. hawaiiense* (McLachlan) + *M. paludicola* Maciolek and Howarth + *M. mauka* Daigle
6. *M. kauaiense* (Perkins) + *M. koelense* (Blackburn)
7. *M. oahuense* (Blackburn) + *M. nesiotes* (Perkins)
8. *M. eudytum* (Perkins) + *M. adytum* (Perkins) + *M. vagabundum* (Perkins) + *M. williamsoni* (Perkins) + *M.* sp. (Kaua'i endemics)

Both phylogenetic analyses supported similar relationships between these subclades, summarized as follows: *Megalagrion* consists of two major clades. The first major clade contains three subclades (1–3) with the *M. xanthomelas* clade (1) diverging first and the

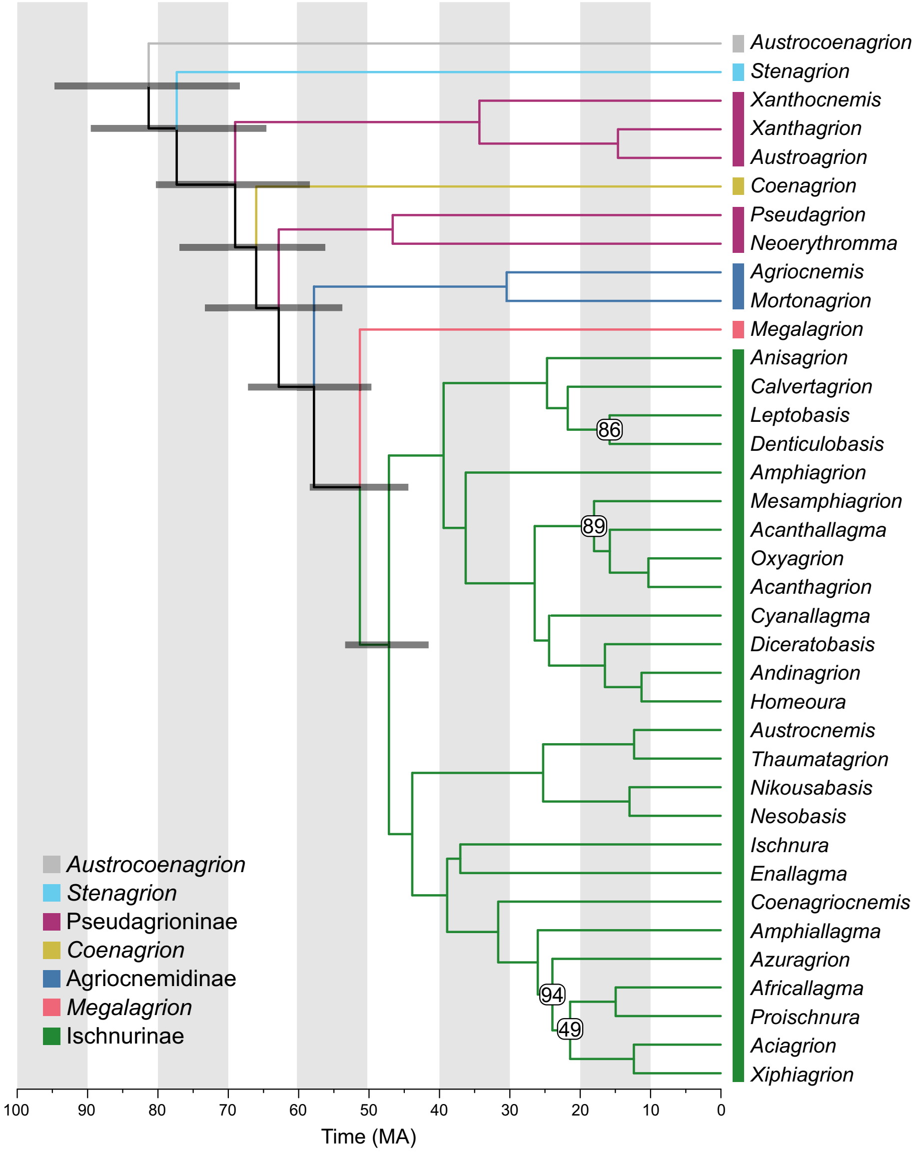

FIGURE 2 Tree shows the topology of the preferred maximum likelihood analysis from the full-length alignment. Ultrafast bootstrap scores are shown for nodes with less than 100% support. Branch lengths reflect divergence times estimated from the preferred BEAST analysis with both fossil and geographic calibration points. Genera are collapsed. *Megalagrion* is labelled, along with subfamilies following (Dijkstra et al., 2014). Grey horizontal bars represent 95% CI for divergence time estimates.

M. orobates clade (2) sister to the *M. oresitrophum* clade (3). The second major clade contains the remaining five subclades (4–8). In the tree generated using a full alignment, the *M. heterogamias* clade (4) diverged first, followed by the *M. hawaiiense* clade (5), but in the tree generated using a target-region-only alignment, the *M. hawaiiense* clade (5) diverged first, followed by the *M. heterogamias* clade (4). Then, in both trees, the *M. kauaiense* clade diverged (6), with the *M. oahuense* clade (7) sister to the *M. eudytum* clade of Kauaʻi endemics (8).

All species with multiple samples were recovered as monophyletic groups except for *M. mauka* and *M. hawaiiense*. One of the samples of *M. mauka* was recovered within *M. hawaiiense*, and the other was recovered as sister to *M. paludicola*.

Species-level relationships were largely the same across both analyses, with exceptions in where *M. kauaiense* was placed and relationships within the clade of Kauaʻi endemics (8). In the tree with a full alignment, *M. kauaiense* was sister to *M. koelense*, forming an ecological clade of damselflies that breed in phytotelmata. However, in the target-region-only alignment, *Megalagrion kauaiense* was within the clade of Kauaʻi endemics (8), sister to *M. vagabundum* + *M. adytum* + *M.* sp. Within the clade of Kauaʻi endemics, *M. adytum* was consistently sister to *M.* sp. and not closely related to *M. eudytum*. However, the placement of *M. vagabundum* and *M. williamsoni* was inconsistent. In the tree with a full alignment, *M. williamsoni* was sister to *M. adytum* + *M.* sp., and *M. vagabundum* was sister to *M. eudytum*. In the tree with a target-region-only alignment, these relationships were swapped, such that

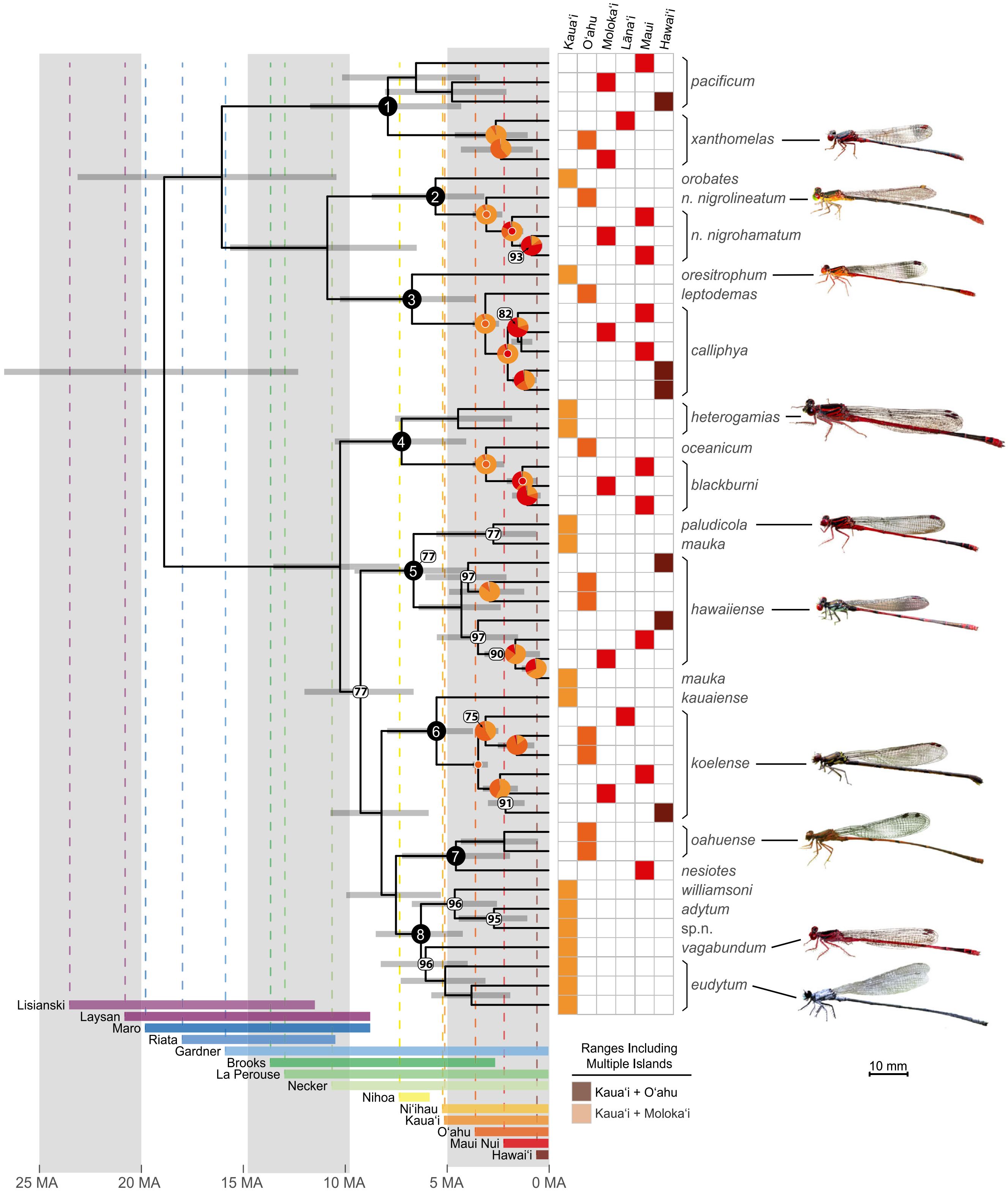

FIGURE 3 Dated phylogeny with fossil and geographic calibration points pruned to *Megalagrion*. Geographically calibrated nodes are marked with a small circle coloured red (Maui Nui) or orange (O‘ahu). Horizontal grey bars represent 95% CI for divergence time estimates. Existence of major Hawaiian Islands above sea level is represented by coloured horizontal bars and emergence times with vertical dotted lines. Current distribution of each taxon represented by coloured squares. Pie charts represent estimated ancestral ranges (coloured by island) and ranges that include multiple islands are coloured according to the legend. Pies are not shown for nodes with an estimated ancestral range that only included area K (Kaua‘i or any other ancestral range) with a probability above 5% or with an estimated ancestral range that was >97% similar to the estimated ancestral range of its most common ancestor. Numbered nodes mark eight clades discussed in the manuscript. Ultrafast bootstrap scores are shown for nodes with less than 100% support. Photo credits: *M. xanthomelas*—Marguerite Butler; *M. n. nigrolineatum*—Nate Yuen; *M. oresitrophum*, *M. heterogamias*, *M. hawaiiense*, *M. vagabundum*—John Abbott; *M. paludicola*, *M. eudytum*—Julien Ueda; *M. koelense*—Idelle Cooper; *M. oahuense*—Kevin Faccenda.

M. williamsoni was sister to *M. eudytum* and *M. vagabundum* was sister to *M. adytum* + *M.* sp.

Relationships within species with more than two samples were somewhat consistent between the two trees, but varied in the following ways:

- *M. xanthomelas*: In the tree with a full alignment the Oʻahu sample was sister to the Molokaʻi sample, but in the tree with a target-region-only alignment the Oʻahu sample was sister to the Lānaʻi sample.
- Both *M. calliphya* and *M. blackburni* had the same pattern: In the tree with a full alignment, the Molokaʻi sample was sister to the West Maui sample, but in the tree with a target-region-only alignment, the Molokaʻi sample was sister to the East Maui sample.
- *M. hawaiiense*: Relationships were consistent except for the Oʻahu samples. In the tree with a full alignment, the Oʻahu samples formed a clade sister to the Hawaiʻi sample from Kohala. In the tree with a target-region-only alignment, the Oʻahu sample from the Koʻolau Mountains was sister to the Hawaiʻi sample from Onomea Stream on the East slope of Mauna Kea, and the Oʻahu sample from Mount Kaʻala was sister to the clade containing the Maui and Molokaʻi samples of *M. hawaiiense* and one of the *M. mauka* samples.
- *M. koelense*: Relationships were consistent except for the samples from Molokaʻi and Lānaʻi. In the tree with a full alignment, the Lānaʻi sample was sister to the Oʻahu samples, and the Molokaʻi sample was sister to the Hawaiʻi sample. In the tree with a target-region-only alignment, the Molokaʻi and Lānaʻi samples formed a clade sister to the Hawaiʻi and Maui samples.
- *M. eudytum*: In the tree with a full alignment, the Kanaele Bog sample was sister to the Kalalau Trail sample from the stream below Hanakāpīʻai Falls. In the tree with a target-region-only alignment, the Kanaele Bog sample was sister to the Kalalau Trail sample taken from the left wall of Hanakāpīʻai Falls.

Species tree and gene tree discordance

The ASTRAL species tree analysis revealed some well supported relationships within *Megalagrion*, especially in the first major clade containing most of the pond-breeding species with foliate gills (clades 1–3) (See Supplementary Information, Figure S2). Clades 1–4 and clade 8 were well supported in the species tree. Species-level relationships were better supported than within-species relationships. There was particularly low support for nodes in the clade containing *M. hawaiiense* + *M. paludicola* + *M. mauka* (clade 8). Gene tree discordance was also high within this clade. The least gene tree discordance was found within clades 2–4, though substantial discordance was found within species and subspecies in the case of *M. nigrohamatum*.

Divergence time estimation

Divergence time estimates with both fossil and geographic calibration points were within the 95% CI of the divergence time estimates with only fossil calibration points but were consistently younger by around 5–10 MY (Table 3). Because our divergence time estimates are generally older than previous estimates, we considered our younger divergence time estimates using both fossil and geographic calibration points to be more conservative, and we used this estimate for downstream analyses. The most recent common ancestor of *Megalagrion* was estimated to be 18.76 million years old (95% CI 12.21–26.57 MA), which is older than the previously estimated 9.6 MA (Jordan et al., 2003) but younger than the 25 MA estimate (Willink et al., 2024). The divergence of *Megalagrion* from Ischnurinae was estimated to have occurred 51.34 MA (95% CI 44.40–58.43 MA), which is consistent with the previous estimate of ~54 MA (Suvorov et al., 2021) (Figure 2, Table 3). The most recent common ancestor of core Coenagrionidae was estimated to be 81.39 million years old (95% CI 64.58–89.54 MA) (Figure 2, Table 3). Within *Megalagrion*, the estimated divergence times of several clades were older than the islands that the clade occupies. For example, divergence times between *M. pacificum* samples all predated the emergence of Oʻahu despite only having samples from the younger islands Maui, Molokaʻi and Hawaiʻi. The divergence time estimate for the *M. calliphya* samples from Hawaiʻi also predated the formation of the island of Hawaiʻi. The younger ends of 95% confidence intervals often included times when younger islands existed, even if the maximum probability estimate near the middle of the 95% CI did not.

From our analysis sampling only from calibration priors without molecular data, we found that 15/16 parameters show substantially narrower posterior than prior distributions (mean width ratio = 0.65), with only one calibration node (*Agriocnemis*) showing equivalent prior and posterior widths (ratio = 1.02), indicating strong agreement between the calibration and molecular data for this node. These findings confirm that (1) fossil and geographic calibrations work independently without problematic interactions, (2) evolutionary parameters are appropriately constrained by the molecular data beyond the calibration priors and (3) the analysis is driven by sequence data rather than dominated by prior assumptions. This validates our calibration strategy and demonstrates that node age constraints do not create unrealistic parameter interactions through the tree structure.

Ancestral range estimation

Results varied depending on the model used (See Supplementary Information, Figure S3). However, the BAYAREALIKE+j model had the best-weighted AIC score (lowest AIC value) (Table S3). Results of this model are shown in Figure 3. Due to the time-stratification, only area K (Kauaʻi and any other unknown ancestral range, including submerged islands) was allowed prior to the emergence of Kauaʻi. Therefore, the ancestral ranges of all nodes with divergence times prior to the emergence of Kauaʻi were necessarily estimated as area K with 100% probability. All eight subclades had a divergence time estimate prior to the emergence of Kauaʻi and thus were estimated with ancestral ranges including only area K. All species were estimated to most likely have an ancestral range including area K.

Ten samples were estimated to most likely have had a most recent common ancestor with an ancestral range other than area K. The common ancestor of *M. nigrohamatum* samples from West Maui and Molokaʻi was estimated to have had an ancestral range of either Molokaʻi (36%) or Maui (32%). The common ancestor of *M. calliphya* samples from Maui and Molokaʻi was estimated to have had an ancestral range of Maui (37%) or Molokaʻi (20%). The common ancestor of *M. blackburni* samples from Molokaʻi and West Maui was estimated to have had an ancestral range of Molokaʻi (36%) or Maui (22%). Finally, the common ancestor of samples of *M. koelense* from Oʻahu and Lānaʻi was estimated to have had an ancestral range of Oʻahu (57%).

There are some surprising results to note, where the ancestral range estimate does not fit the progression rule hypothesis, or is unintuitive. For example, although we included no samples of *M. pacificum* from Kauaʻi, the ancestral range of the species was estimated as area K. This was a common pattern across the tree, where species that were expected to have an ancestral range of Oʻahu or Maui Nui were instead estimated to have an ancestral range of area K, even when the estimated divergence time of the species did not predate the formation of younger islands such as Oʻahu or Maui Nui. In general, ancestral range estimates favored distributions that included older areas. A limitation of the BioGeoBEARS analysis we conducted was the unrealistic precision of divergence times at each node, since each node was given a single date rather than a probability distribution.

Diversification and extinction

Using RPANDA, we analysed diversification rate heterogeneity across 11 clades (including the backbone) representing major lineages within Coenagrionidae. Crown shifts were strongly preferred over stem shifts (ΔAICc = 39.4), indicating that rate changes occurred at crown nodes affecting only descendant lineages rather than entire subclades including stem branches. The best-supported model identified seven clades with significant diversification rate shifts relative to the family-wide backbone: Pseudagrioninae, Agriocnemini, Ischnurinae, Nesobasini, Acanthagrionini, Megalagrion and Teinobasinae (See Supplementary Information, Table S4) (AICc = 2137.3, 19 parameters). Among these shifted clades, five exhibited constant speciation rates (BCST models) while two showed time-varying speciation rates (BVAR models): Agriocnemini with accelerating rates through time ($\alpha = 0.070$) and Megalagrion with accelerating rates through time ($\alpha = 0.224$). Nesobasini displayed the most extreme parameter values among all shifted clades, with an initial speciation rate of $\lambda = 0.245$ events per lineage per million years, representing a + 0.095 increase relative to the backbone rate ($\Delta\lambda = +0.095$) (See Supplementary Information, Table S5). In contrast, *Megalagrion* and Agriocnemini showed the lowest initial speciation rates ($\lambda = 0.060$ and $\lambda = 0.080$, respectively), with substantial negative deviations from backbone parameters ($\Delta\lambda = -0.090$ and $\Delta\lambda = -0.069$). The remaining shifted clades showed intermediate rate patterns, with most displaying modest increases or decreases relative to backbone diversification rates.

Simulation modelling

The geographic submodel of our agent-based simulation model produced island chains with similar characteristics to the Hawaiian Islands with some variation (Table 6). The average number of extant species at the end of each run was also similar to the number of species of *Megalagrion* that currently exist (24.74 species simulated vs. 23 species *Megalagrion*), yet was highly variable (standard deviation: 25.85 species). The average total number of species created was very large (1286.07 species). It should be noted that in the model, every population lineage with a distinct niche (determined by an equation and threshold in the model and described in the ODD (Supplementary methods)) is regarded as a separate species and that these species are recorded regardless of how long they survive. According to these results, the average speciation and extinction rates were both high, yet the net diversification rate was moderately positive. Manual visual inspection of phylogenetic trees produced by the model indicated periods of rapid speciation and extinction, separated by periods of stability with little diversification (See Supplementary Information, Figure S4).

Ancestral state reconstruction

The best evolutionary transition rate model based on AIC score was an equal rates model for reconstructing breeding habitat and a symmetric rates model for reconstructing gill type. These models were used to perform reconstruction analyses. Ancestral state reconstruction results using flat priors in the case of missing data are shown in

TABLE 6 Simulation model results.

	Island size (km^2)	Number of islands	Length of island chain (km)	Extant species	Total species created	Extinction rate (species/my)	Speciation rate (species/my)	Net diversification rate (species/my)
Mean	2878	8.36	821.02	24.74	1286.07	−630.67	643.04	12.37
Standard deviation	311	0.45	36.88	25.85	1654.93	−814.54	827.47	12.93
Empirical data from current Hawaiian Islands	2080	8	630	23				

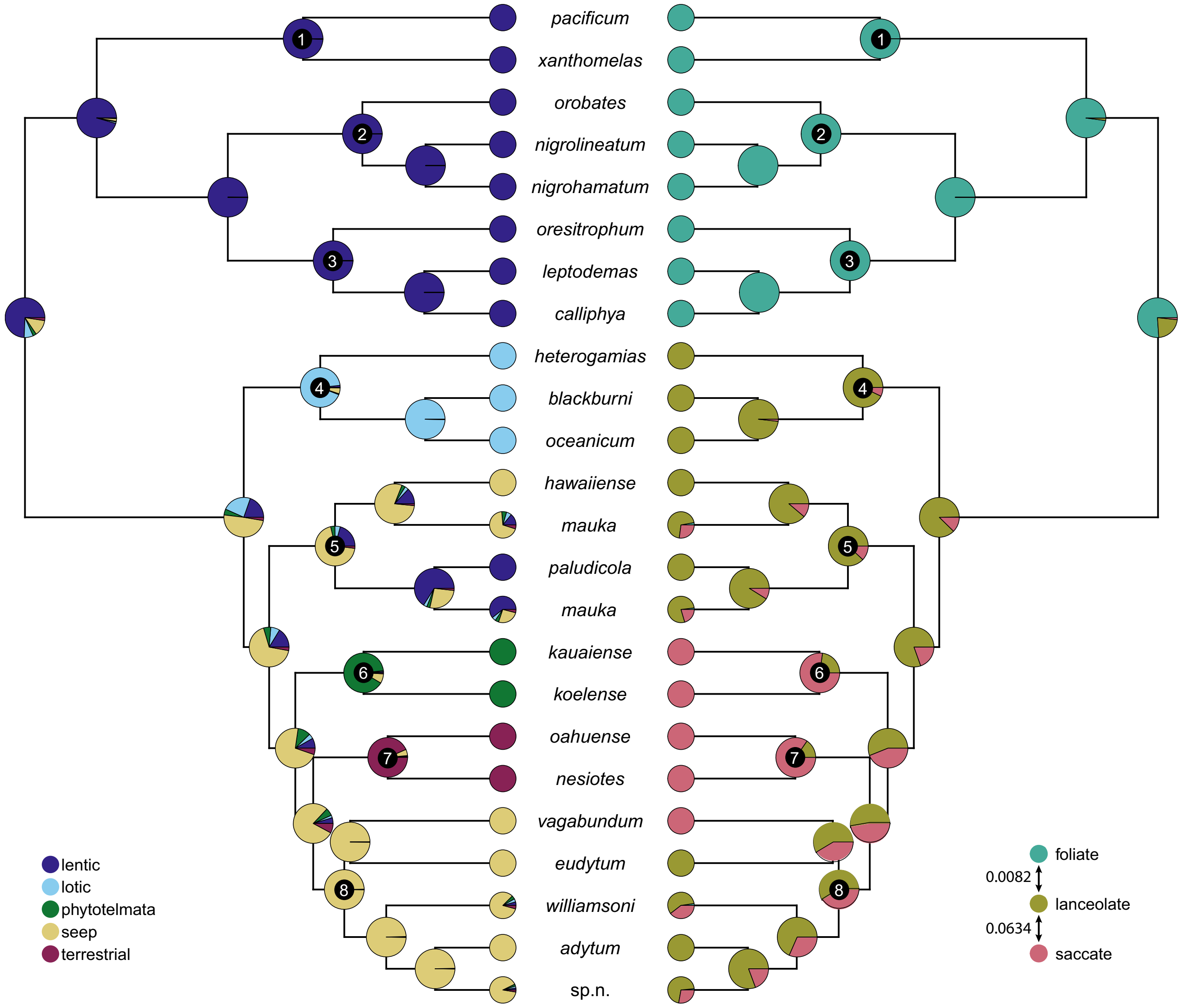

FIGURE 4 Ancestral state reconstruction of breeding habitat (left) and gill type (right) using the best evolutionary transition rates model (equal rates for breeding habitat, symmetric rates for gill type). Transition rates for gill type are shown in the legend (transition rate between foliate and saccate is 0). Each tree is a summary of 10,000 stochastic character mappings using flat priors for unknown character states for *M. mauka*, *M. williamsoni* and *M.* sp. Pie charts on nodes (and tips for *M. mauka*, *M. williamsoni* and *M.* sp.) represent the proportion of simulations that recovered the node as each character state. Numbered nodes mark eight clades discussed in the manuscript.

Figure 4. Both methods for dealing with missing data (flat priors, taxon removal) reconstructed the common ancestor of *Megalagrion* as a species that bred in lentic waters with foliate gills (See Supplementary Information, Figure S5).

The major clade containing the *M. pacificum*, *M. oresitrophum* and *M. orobates* subclades (1–3), as well as all clades contained within it, was also reconstructed with an ancestor breeding in lentic waters with foliate gills regardless of method. The method of analysis did not change the most likely reconstruction for breeding habitat at any node. The common ancestors of each subclade (1–8) were reconstructed as breeding in the habitat occupied by their constituents, even in the cases with missing data (*M. hawaiiense* and *M. eudytum* clades (5 and 8)). The most likely reconstruction for the common ancestor of all other larger clades was reconstructed as a species breeding in seeps, though with less certainty at the node of the major clade containing all species that do not breed in lentic water.

The most recent common ancestors of the phytotelmata- and terrestrial-breeding clades were reconstructed with saccate gills. The most recent common ancestors of the lotic-breeding clade (4) and the seep-breeding *M. hawaiiense* clade (5) were reconstructed with lanceolate gills. Depending on the method used for dealing with missing data, nodes where the phytotelmata-breeding clade (6), terrestrial-breeding clade (7) and *M. eudytum* clade of Kaua'i endemics (8) diverged were reconstructed as either having lanceolate or saccate gills. Eliminating the species with missing data (*M.* sp. and *M. williamsoni*) or using a flat prior for the gill type of these species resulted in a near-equal probability reconstruction of either lanceolate or saccate gills for the most recent common ancestor of the *M. eudytum* clade of Kaua'i endemics (8) and a slightly greater probability of saccate gills for the nodes where the phytotelmata-breeding clade (6) and terrestrial-breeding clade (7) diverged. A reconstruction with a lanceolate-gilled ancestor at these nodes suggests convergent evolution of saccate gills up to three times, in *M. vagabundum* and the phytotelmata- and terrestrial-breeding clades (6 and 7). A reconstruction with a saccate-gilled ancestor at these nodes suggests the convergent reversal to lanceolate gills at least twice in *M. eudytum* and *M. adytum*. Various additional combinations of gains and reversals are also possible given these results. One such sequence includes the initial evolution of saccate gills prior to the divergence of the phytotelmata-breeding clade (6), convergent reversal to lanceolate gills in the common ancestor of *M. eudytum* and *M. vagabundum* and in *M. adytum*, and convergent evolution of saccate gills in *M. vagabundum*.

DISCUSSION

The findings presented in this study significantly advance our understanding of the phylogenetic relationships, temporal origins, biogeographic history and diversification patterns of the Hawaiian *Megalagrion* damselflies. By leveraging targeted genome-wide sequencing and comprehensive taxon sampling, we have provided robust phylogenetic hypotheses that clarify relationships within *Megalagrion* and provide support for the phylogenetic position of *Megalagrion* within Coenagrionidae.

Our results add to the evidence for the sister relationship between *Megalagrion* and Ischnurinae, as well as the sister relationship between Agriocnemidinae and Ischnurinae + *Megalagrion* (Dumont et al., 2010; Suvorov et al., 2021; Willink et al., 2024). Members of the Ischnurinae and Agriocnemidinae have distributions all over the world, leaving the geographic origin of *Megalagrion* a mystery.

Origin of Hawaiian damselflies

The gap in timing between *Megalagrion*'s divergence from the other coenagrionids over 50 MA and from its most recent common ancestor approximately 19 MA leads us to hypothesize that *Megalagrion* has evolved for tens of millions of years prior to its current radiation, isolated from any other populations of damselflies. Early *Megalagrion* ancestors perhaps dispersed down the Hawaiian Island chain as islands emerged and submerged. However, a lack of any significant islands 33–29 MA (Figure 1) is hypothesized to limit the maximum estimated age of most Hawaiian biota to 29 MA (D. A. Clague et al., 2010). Therefore, island hopping is most likely to have occurred more recently than 29 MA with the emergence of the first Northwestern Hawaiian Islands (i.e., from Nihoa to Kure), and with increasing likelihood over time as additional high islands such as Laysan and Gardner formed 21 MA and 16 MA, respectively. These high islands would have provided a variety of perennial freshwater habitats for colonization. The explanation for how and where *Megalagrion* ancestors evolved prior to 29 MA is unknown, perhaps existing on some other isolated Pacific Island, having extinct ancestral populations on a continental mainland, or managing to survive and disperse across tiny, low-elevation, ephemeral islands between the Emperor Seamount chain and the Northwest Hawaiian Islands. Future comparative-genomic studies may aid in recovering ancient population dynamics in *Megalagrion* that could test hypothesized bottlenecks and founder events due to significant dispersal events.

Extinction in the early evolution of *Megalagrion*

In our RPANDA analysis, *Megalagrion* was identified as one of seven clades showing significant rate shifts relative to the backbone. This shift was characterized by low initial speciation rates compared to the backbone ($\lambda = 0.060$, $\Delta\lambda$ from backbone $= -0.090$) and strong temporal acceleration in diversification ($\alpha = 0.224$, $\Delta\alpha$ from backbone $= +0.242$). This pattern suggests that while *Megalagrion* began with substantially lower speciation rates compared to other coenagrionid lineages approximately 51 MA, diversification rates increased dramatically through time, particularly during the recent radiation across the Hawaiian Islands beginning approximately 19 MA.

The low initial speciation rate in *Megalagrion* may reflect the challenges of establishing and diversifying on isolated oceanic islands, while the subsequent strong acceleration corresponds to adaptive radiation into available ecological niches and dispersal to newly emergent islands. Extinction may have occurred in the early evolution of Megalagrion, though our RPANDA analyses provide no evidence for this. The gap between the divergence of *Megalagrion* from Ischnurinae and the most recent common ancestor of extant *Megalagrion* suggests a prolonged period during which extinction could have eliminated early lineages. This process could produce extant species with an identical phylogenetic topology to the low speciation rate suggested by the best performing RPANDA model. The dynamic geologic history of the Hawaiian–Emperor chain, with islands repeatedly emerging and submerging over tens of millions of years, would have created numerous opportunities for local and regional extinctions.

Results from our simulation model provide additional insight into possible diversification patterns on an oceanic hotspot island system like the Hawaiian Islands. The model estimated that over 20 million years, speciation outpaced extinction by a small margin, yielding a moderately positive net diversification rate. Although “species” taxa in the simulation are not directly comparable to *Megalagrion* species in nature, it is interesting to note that the average number of extant species at the end of the simulation (24.74 species after 20 MY) is similar in number to the number of extant species in *Megalagrion* after a similar amount of time since its most recent common ancestor (23 species after 19 MY). The simulation's results support the hypothesis that extinction played a significant role in the evolution of *Megalagrion* and that multiple lineages related to *Megalagrion* have gone extinct in the past 20 million years. Extinction during this period is expected due to the submergence of large islands such as Lisianski, Laysan, Maro, Riata, Brooks and Gardner (Price & Clague, 2002). This result is likely not unique to Megalagrion, and we suspect that other coenagrionid lineages across the Pacific may have experienced similar diversification patterns with significant extinction. However, there is little empirical data on past extinction rates in Odonata that can be compared to our simulated patterns. Therefore, our ability to draw conclusions from this simulation experiment is limited.

Based on our combined results, we hypothesize that after *Megalagrion* diverged from Ischnurinae about 51 MA, *Megalagrion* diversification rates were low, either due to decreased speciation rate, increased extinction rate or both. Around 19 MA, *Megalagrion* colonized the Northwestern Hawaiian Islands and diversified and dispersed across the island chain. As these older islands subsided, some lineages went extinct while at least one lineage successfully dispersed to the emerging current Hawaiian Islands, giving rise to the 23 extant species of *Megalagrion* and likely additional species that have since gone extinct. The recent radiation of *Megalagrion* in the last 19 million years is comparable in numbers of species to other similarly aged lineages within Coenagrionidae, suggesting that at least for the past 19 million years, speciation rates have not been lower than expected. This is supported by both our RPANDA and simulation models and aligns with findings in other endemic Hawaiian lineages, as speciation rates of Hawaiian fauna are often similar to or greater than those seen in mainland counterparts (Baldwin et al., 1991; Bonacum et al., 2005; Cowie, 1992; Cowie & Holland, 2008; Givnish et al., 2009; Haines et al., 2014; Hembry et al., 2021; Johns et al., 2018; Johnson et al., 1989; Landis et al., 2018; Lindqvist & Albert, 2002; O'Grady et al., 2011; Otte, 1994; Régnier et al., 2015; Roderick & Gillespie, 1998; Rubinoff & Schmitz, 2010; Rundell et al., 2004; Russo et al., 1995). In addition, phylogenetically distinct populations within species such as *M. nigrohamatum*, *M. koelense*, *M. hawaiiense*, *M. pacificum*, *M. xanthomelas* and *M. calliphya*, and within the recent *M. eudytum* clade of Kauaʻi endemics (8) suggest that speciation both within and between islands is ongoing. This is in line with previous population studies within *M. xanthomelas*, showing a high degree of genetic differentiation between populations from Maui Nui and those from the island of Hawaiʻi (Jordan et al., 2005, 2007).

Ecological diversification

Our diversification results for *Megalagrion* can be explained in two phases: ecological diversification, which took place in ancestral islands, and spatial diversification, which takes place on the current Hawaiian Islands (Figure 3). In the first phase, ecological diversification likely occurred in the Northwestern Hawaiian Islands prior to the emergence of Kauaʻi, giving rise to at least five ecologically distinct lineages that breed in different habitats. Speciation within the lentic-breeding and seep-breeding clades gave rise to three additional lineages, possibly prior to the emergence of Kauaʻi. These early diversification events account for approximately ⅓ of species diversity within *Megalagrion*, including all eight subclades except for the terrestrial-breeding subclade (7). Aside from the extant representatives of these lineages, additional members of these clades, especially single-island endemics, as we see in the extant *Megalagrion*, or additional clades altogether, may have existed but not managed to disperse from the Northwestern Hawaiian Islands to the current Hawaiian Islands. For example, the terrestrial-breeding subclade is the only ecological subclade without a representative on Kauaʻi. We hypothesize that the terrestrial-breeding subclade diverged on Kauaʻi or an older island, but that the Kauaʻi representative of this subclade is now extinct, leaving terrestrial-breeding species from only Oʻahu, Maui Nui and Hawaiʻi, and an artificially young age for this clade. This hypothesis is supported by investigations into the conservation concern of *Megalagrion*. Terrestrial-breeding species of *Megalagrion* may be more vulnerable to extinction than species breeding in other habitats and are currently of high conservation concern (Englund et al., 2007).

Our results indicate that the seep habitat is likely the first novel habitat that *Megalagrion* entered, and all species that occupy additional novel habitats evolved from seep-breeding ancestors (Figure 4). This seep-breeding ancestor also evolved lanceolate gills. These results support the hypothesis that morphological adaptations gained from breeding in seeps facilitated adaptation to lotic-breeding habitats (Polhemus, 1997). Specialization to a specific habitat has been shown to alter the adaptive landscape of a lineage, allowing for new possible evolutionary trajectories (Kolmann et al., 2020). Adaptation to breeding in seeps may have also facilitated adaptation to the other novel breeding habitats in *Megalagrion*. Seep habitats often contain a variety of microhabitats and are physically adjacent to a variety of additional habitats. Specialized adaptations to these microhabitats may have facilitated evolutionary jumps to adjacent novel habitats. Specialization within the seep habitat may also have been responsible for within-island speciation, giving rise to the *M. eudytum* clade of seep-breeding species endemic to Kauaʻi. As these speciation events likely occurred within the same island and within seep habitats, some of these speciation events may have occurred sympatrically. Species within this clade can be found living nearby one another but occupying separate microhabitats. The species in this clade are all classified as breeding in seeps, both in our analysis and in previous analyses by Polhemus (1997) and Jordan et al. (2003). However, field observations of these species' breeding habitats indicate specialization within the seep habitat (Polhemus & Asquith, 1996). For example, *M. adytum* and *M. eudytum* both breed in rheocrenes, but *M. adytum* prefers wet walls bordering headwaters in Alakaʻi swamp, whereas *M. eudytum* prefers seeping cliffs below the plateau. These specializations may be a result of speciation through the partitioning of rheocrene niches within Kauaʻi. However, these hypotheses remain largely untested. Efforts to understand and characterize the niches of members of this clade, as well as increased taxon sampling in phylogenetic and population-genetic studies of this clade, may reveal the mechanisms of speciation that gave rise to this mini-radiation.

It is noteworthy that all of the major ecological innovations within *Megalagrion* may have evolved prior to the existence of the current

Hawaiian Islands and that recent speciation, aside from possible niche partitioning on Kauaʻi, has been primarily due to dispersal rather than ecological diversification. It may be that by the time *Megalagrion* began to disperse across the current Hawaiian Islands, the damselflies had already begun to exploit most available niches, leaving little available niche space to expand. As representatives from each ecological clade dispersed across the islands, they colonized these niches and competitively prevented re-colonization.

Several species endemic to Kauaʻi present possible exceptions to the pattern that ecological diversification predates the current Hawaiian Islands. Several of these species are associated with bog habitats in the Alakaʻi Swamp and Kanaele Bog. These bog habitats developed under specific geologic conditions present on Kauaʻi, specifically a relatively level plateau of dense, less porous lava where rainfall exceeds drainage (Sohmer & Gustafson, 1994; Van't Woudt & Nelson, 1963). It is not known if similar bog habitats ever formed on the older Northwestern Hawaiian Islands. If bog habitats were not present on these older islands, then it is unlikely that any ecological adaptation to bog habitats in *Megalagrion* pre-dated colonization of Kauaʻi. Some patterns of speciation are complex, including within the *M. hawaiiense* + *M. mauka* + *M. paludicola* clade. The position of *M. mauka* has never been clearly resolved. Polhemus (1997) found *M. mauka* in a polytomy with *M. paludicola* and *M. eudytum*, and Jordan et al. (2003) found paraphyly of *M. mauka* with respect to *M. paludicola*. Here, we find *M. mauka* paraphyletic with high gene tree discordance and low support in both ultrafast BS in our concatenated maximum likelihood analysis and local posterior probabilities in our coalescent-based ASTRAL analysis, despite having acceptable coverage across all loci. Species delimitation in this clade remains an issue and likely reflects the real biological complexity of introgression, hybridization, sympatric speciation and/or cryptic species.

Diversification by dispersal to new islands

In the second major phase of diversification in *Megalagrion*, speciation occurred within each of eight subclades as representatives from each clade dispersed to new islands. Our results indicate that these dispersal events largely follow the progression rule, where older species occupy older islands and younger species occupy younger islands. However, dispersal between the younger islands of Maui Nui and the island of Hawaiʻi does not appear to follow any particular pattern. Moreover, phylogenetic relationships between species on the islands of Maui Nui and Hawaiʻi vary depending on which sequencing dataset was used, making it difficult to test specific hypotheses regarding the timing and direction of dispersal. Patterns of dispersal within the *M. hawaiiense* clade are particularly interesting, with evidence of back-dispersal from the island of Hawaiʻi to older islands. In future phylogenetic and population-genetic studies, increased sampling of members of the *M. hawaiiense* clade, as well as species distributed across Maui Nui and Hawaiʻi, should be attempted to test dispersal hypotheses.

We suspect that speciation both due to isolation between the islands of Maui Nui and Hawaiʻi and due to niche partitioning on Kauaʻi is ongoing. Related populations on the islands of Maui Nui have been recently isolated from one another for as little as 20 KA (Price & Elliott-Fisk, 2004); populations that dispersed from Maui Nui to Hawaiʻi have been isolated for less than 600 KA (D. Clague, 1996).

Some patterns of diversification in *Megalagrion* seem to coincide with previous broader findings that show wide ecological and morphological diversification early in the evolutionary history of a clade as well as bursts of morphological diversity on isolated branches with a sudden increase in rate (Cooney et al., 2017). Within a relatively small radiation of insects in terms of the number of species, *Megalagrion* showcases many observed patterns of evolution seen in other insect radiations in the Hawaiian Islands (Hembry et al., 2021). These patterns include colonization of now-sunken islands followed by dispersal to islands as they emerge, ecological and morphological diversification through adaptive radiation, speciation due to dispersal to different islands and speciation within islands and speciation largely but not completely following the island age progression rule.

CONCLUSION

The findings presented in this study significantly advance our understanding of the phylogenetic relationships, temporal origins, biogeographic history and diversification patterns of the Hawaiian *Megalagrion* damselflies.

Our results support the following hypothesized patterns of evolution and diversification in *Megalagrion*: The ancestor of *Megalagrion* diverged from Ischnurinae over 50 million years ago, leading to multiple lineages. At least one of these colonized one of the Northwestern Hawaiian Islands around 19 million years ago (perhaps Lisianski, Maro or Laysan). This lineage diversified into several ecological niches with associated adaptations in gill morphology on larger islands like Gardner. Several, if not all, of these lineages managed to disperse across the Northwestern Hawaiian Islands to Kauaʻi. Each of these successful colonists dispersed across the current Hawaiian Islands as they emerged, leading to island-endemic species from each ecological clade. An island-endemic representative of the terrestrial clade may have existed on Kauaʻi that has since gone extinct. On Kauaʻi, adaptation to new niches, including bogs and further niche partitioning, drove additional diversification within the island. Speciation in *Megalagrion* is ongoing as populations from the most recent island of Hawaiʻi diverge from their closest relatives from the islands of Maui Nui.

The geographic origin of *Megalagrion* is still unknown, and hypothesized extinction in the early evolution of *Megalagrion* remains empirically untested. Comparative genomics including several species of *Megalagrion* may provide insight into potential bottleneck events or selective pressures that have occurred throughout the evolution of *Megalagrion*.

Higher resolution patterns of diversification within clades or even species of *Megalagrion* require further investigation and may yield additional insights into evolutionary and biogeographic processes on islands, namely, patterns of population-level diversification within more widespread species (both across the Hawaiian Islands and within Maui Nui and Hawai'i) and patterns of diversification within the island of Kaua'i. Within these species, various taxonomic issues persist that will need to be resolved for effective experimental design and hypothesis testing. Several island-endemic populations were originally described as separate species but have since been synonymized due to a lack of stable diagnostic morphological characters (Blackburn, 1884; Kennedy, 1934; Perkins, 1899; Polhemus & Asquith, 1996; Williams, 1936; Zimmerman, 1948). These taxonomic hypotheses should be evaluated with molecular and ecological data, as well as additional morphological data to determine if populations or subspecies should be elevated or redeemed to species status. In addition, the conservation of threatened species and populations within *Megalagrion* is important for allowing further investigation and relies on sound taxonomy.

Investigations focusing on describing and comparing the niches of species on Kaua'i may increase our understanding of niche partitioning or sympatric speciation. Investigating additional morphological systems as potential adaptations to ecological niches may improve our understanding of adaptive radiation. Potential adaptations may be present in female ovipositors, eyes (Scales et al., 2016), coloration (Cooper, 2010), body size and more.

The Hawaiian damselflies of the genus *Megalagrion* have been and continue to be a fruitful study system for understanding evolution, diversification and biogeography. We expect additional insights in these fields as further investigation is conducted.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest in relation to this work.

DATA AVAILABILITY STATEMENT

Data, scripts and results relating to this study can be found in a dryad repository at DOI: https://doi.org/10.5061/dryad.18931zd80 (Hadfield, 2025). Sequences used for this project can be found in the dryad repository in the folder trees_from_cluster/core_coenagrionidae_full_sampling/probe_orthologs and are currently being submitted to NCBI under the following BioProject: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1265769.

ETHICS STATEMENT

This study did not involve any experiments with live animals or human subjects that would require formal ethics approval. Fieldwork, including the collection of samples, was conducted in accordance with the relevant permitting requirements, as authorized by the Department of Land and Natural Resources of the State of Hawaii, endorsement no. I5420.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

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Robert K. Hadfield: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; software; validation; visualization; writing—original draft; writing review and editing. **Steve Jordan**: Conceptualization; resources; writing—review and editing. **Dan A. Poihemus**: Conceptualization; methodology; project administration; resources; supervision; writing—review and editing. **Laura N. Sutherland**: Data curation; investigation; project administration; writing—review and editing. **Steven L. Peck**: Conceptualization; methodology; supervision; writing—review and editing. **John C. Abbott**: Data curation; resources; writing—review and editing. **Paul B. Frandsen**: Data curation; methodology; resources; software; supervision; writing—review and editing. **Robert P. Guralnik**: Conceptualization; methodology; resources; writing—review and editing. **Vincent J. Kalkman**: Data curation; resources; writing—review and editing. **Jessica L. Ware**: Data curation; funding acquisition; resources; writing—review and editing. **Seth M. Bybee**: Conceptualization; data curation; funding acquisition; investigation; project administration; resources; supervision; writing—review and editing.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Full and target maximum likelihood tree comparison.

Figure S2. Gene tree cloudogram and ASTRAL species tree.

Figure S3. BioGeoBEARS results.

Figure S4. Example of simulated phylogeny.

Figure S5. Ancestral state reconstructions.

Data S1. Supporting information.

Table S1. Taxon Sampling.

Table S2. Extended RPANDA taxon sampling.

Table S3. Comparison of BioGeoBEARS models.

Table S4. RPANDA best models.

Table S5. RPANDA parameter comparison.

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