THE EFFECTS OF HABITAT SPECIALIZATION ON POPULATION STRUCTURE IN HAWAIIAN DAMSELFLIES

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ABSTRACT

Hawai‘i is a diverse ecological hotspot of biodiversity, home to many adaptive radiations including a clade of damselflies which encompass the full known range of damselfly breeding habitats and encompass a range of habitat specificity but are of increasing conservation concern. I tested the effects that habitat specificity might have on gene flow and population differentiation between a relative generalist *Megalagrion vagabundum* and a relative specialist *Megalagrion nigrohamatum nigrolineatum*. Mitochondrial genes indicate that there is significant differentiation at a fine-scale in *M. vagabundum* and suggests that differentiation may be even stronger in *M. n. nigrolineatum*. These data are further discussed with respect to genetic variation within these two species and possible barriers to dispersal and the ecology and conservation of these two *Megalagrion* species.

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Introduction

Understanding the connectivity among populations of a species provides key insights into micro-level evolutionary processes. For instance, a greater degree of isolation between populations should provide opportunities to evolve differences among them (Wright, 1951; Lowe and Allendorf, 2010). When natural selection is involved across a complex landscape, these populations may undergo a process of local adaptation that may eventually lead to speciation. Alternatively, increased gene flow between populations will result in admixture and homogenization of the population which can counteract local adaptation but is necessary for spreading highly advantageous alleles, maintaining genetic diversity and lowering the risk of inbreeding depression and local extinction, particularly for small populations (Frankham, 1995; Saccheri *et al*., 1998; Rieseberg and Burke, 2001). The extent of movement and gene flow between distinct populations can be inferred through patterns of neutral genetic differentiation (Slatkin, 1987).

There are many factors that can contribute to gene flow within a species, one of the most significant and well-studied is dispersal, or the ability of an individual from one subpopulation to reach another subpopulation and pass along its genes (Bohonak, 1999). Habitat specialization is not as well studied, but it can promote population subdivision as it is often associated with limited dispersal capability (see Roff, 1990). Habitat specificity also plays a large role in population structure regardless of dispersal ability (e.g. Rocha *et al*., 2002; Marko, 2004; Ayre *et al.*, 2009). Even if a species is able to reach a new location, the ability to establish at this new habitat is a function of how the species interacts with the environment. As a consequence, species which are eurytopic are more likely to establish in more habitats regardless of whether they are better at dispersing. Because of the combined effects of dispersal and habitat specificity, an intriguing possibility is that the evolution towards habitat specialization itself may promote differentiation and speciation.

Dispersal in dragonflies and damselflies (Odonata) is relatively well studied due to the ease at which mark-release-recapture studies can be done in this group (reviewed in Cordoba-Aguilar, 2008). Damselflies (Zygoptera) have the potential to be good dispersers, but are generally philopatric with patchy distributions (Conrad *et al.*, 1999; Geenen *et al.*, 2000; Angelibert and Giani, 2003; Purse *et al.*, 2003). Some species have extremely specific microhabitat requirements (e.g. *Nehalennia speciosa*, Bernard and Schmitt, 2010; *Megaloprepus coerulatus*, Fincke and Hedstrom, 2008) whereas other species are more general and can be found wherever there is adequate water and sunlight (Orr, 2004). Habitat specificity is found to have a significantly negative effect on dispersal capability in odonates

whereas wingspan and habitat type (lentic versus lotic) do not show any predictive effects on dispersal (Harabis and Dolny, 2011).

Megalagrion damselflies represent an ideal system to test the effects of habitat specialization on population structure. They are a diverse clade of 23 species that exploit an impressive range of breeding habitats including: streams, pools, seeps, phytotelmata, and fully terrestrial (Polhemus and Asquith, 1996; Jordan *et al.*, 2003) with many species presenting adaptations and preferences to specific microhabitats (Jordan *et al.*, 2003; Scales and Butler, 2016; Henry *et al*., 2017; Rivera *et al.,* in prep). Studies on *Megalagrion* gene flow and genetic diversity have revealed that populations are not connected across ocean channels (Jordan *et al*., 2005; Jones and Jordan, 2015) and there may be variation in genetic diversity within species between islands (Jordan *et al.*, 2007). But there have been no tests for within-island population subdivision in any Hawaiian damselfly species despite conservation concern and known occurrences of within-island speciation.

Two species that differ in habitat specificity are the relatively more specialized blackline Hawaiian damselfly, *Megalagrion nigrohamatum nigrolineatum* (*M. n. nigrolineatum*), and the relatively more generalized scarlet Kaua‘i damselfly, *Megalagrion vagabundum* (*M. vagabundum*). *M. n. nigrolineatum* is a pool-breeding specialist which spends the majority of its time perched along dark slow-flowing montane stream tributaries and pools along streams (Polhemus and Asquith, 1996; Henry *et al*., 2017). The habitat in which it lives is cluttered with vegetation and receives much less light than habitats where congeners are usually found (Henry *et al.*, 2017; Rivera *et al*., in prep). *M. vagabundum* is a generalist that can be found by seeps and a variety of habitats, it is also more likely to be seen far away from breeding sites in open areas and is more adaptable to different breeding habitats, including a manmade leaking cane flume (Polhemus and Asquith, 1996). In addition to habitat type, light intensity is extremely ecologically important in *Megalagrion* and seems to be a key indicator of habitat preference and habitat suitability in many odonates in general (Rivera *et al.*, in prep.; Henry *et al.,* 2017; Saito *et al*., 2016, Remsburg *et al*., 2008; Pezalla, 1979). Light preferences are taxon-dependent, *M. vagabundum* has demonstrated a much wider range of light environmental tolerances whereas *M. n*. *nigrolineatum* has a highly specialized preference for extremely dark habitats (Rivera *et al*., in prep, Henry *et al.*, 2017).

Knowledge of their population genetic structure should be valuable for conservation and management as well as understanding the genetic variation and history of these species. Our knowledge of *M. n. nigrolineatum* and *M. vagabundum* distributions are based solely on stream site presence and absence surveys. Both species are endemic to a single island and are of

conservation concern. *M. n. nigrolineatum* is endemic to O‘ahu and is listed as endangered. Population sizes in *M. n. nigrolineatum* are presumed to be very small (1000 individuals total; Polhemus, FR) while *M. vagabundum* is endemic to Kaua‘i and is IUCN listed as vulnerable.

The diversification of available habitat as Hawaiian Islands age and the inability to cross open ocean channels are known to be major drivers in the evolution of *Megalagrion* species, but the effects of habitat specificity have not been explored despite some species being quite stenotopic (Jordan *et al.*, 2003). If habitat specialization is driving population differentiation, we expect *M. n. nigrolineatum* to have greater genetic differentiation between populations while *M. vagabundum* would have low genetic differentiation between populations. Furthermore, we also quantified genetic diversity in both species to contribute to our understanding of demographic histories and the conservation needs for both damselflies. Population genetic analysis may help to identify demographically independent (and possibly locally adapted) sets of populations that may require specialized management attention (Moritz, 1994; 2002).

Methods

Study Populations and Sampling

We studied multiple damselfly populations of two species on O‘ahu and Kaua‘i (Figure 1). The populations were chosen to span potential factors in genetic isolation: geographic distance as well as mountain ridges. On O‘ahu, *M. n. nigrolineatum* (hereafter, *M.n.n*.) was collected throughout its range in the Ko‘olau mountains, with two populations on the leeward side of the main ridge and three on the windward side. The leeward populations were Mānoa valley along the ‘Aihualama stream (21°20.123'N, 157°48.167'W) and Poamoho ridge (21° 32.011'N, 157° 55.508'W). The windward populations included Waiāhole stream (21° 29.400’N, 157° 52.967’W), Ma‘akua stream (21° 35.055’N, 157° 55.475’W), and Kaipapau stream (21° 35.499'N, 157° 55.926'W). Sample sizes are given in table 1.

On Kaua‘i, *M. vagabundum* (hereafter, *M.v*.) was collected across four different sites around the main shield volcano of the island. Individuals were caught along Makaleha stream (22° 06.245’N, 159° 23.640’W), Wailua River North Fork (Wailua R N Fork; 22° 03.890’N, 159° 28.401’W), Kawaikoi stream (22° 08.178’N, 159° 36.773’W), and along the Hono O Nā Pali trail (Nā Pali; 22° 12.025’N, 159° 36.468’W). Damselflies were collected with hand-sweep nets between the years of 2011-2014 using state and local permits (FHM10-235, FHM13-300, FHM14-337, ODF-111711R, FR-120616-01). Population sample sizes were limited by the difficulties of collecting rare organisms over difficult terrain and by permit collection limitations.

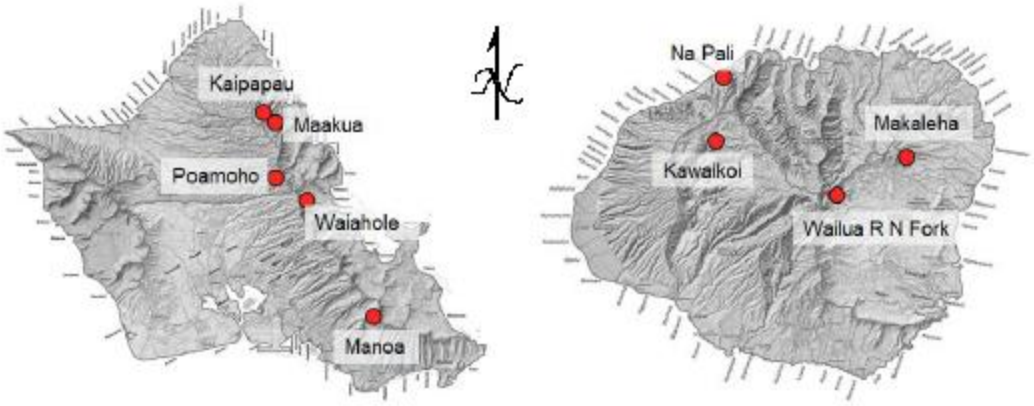

Figure 1. a) sampling sites for *M. n. nigrolineatum* on O‘ahu. b) sampling sites for *M. vagabundum* on Kaua‘i

DNA Extraction and Sequencing/Genotyping

DNA was extracted for sequencing and genotyping from insect leg tissue. Damselfly specimens had been previously frozen in air and stored at -20^{o}C and -80^{o}C. DNA was extracted from leg muscle using a Qiagen DNEasy kit. Because legs are covered with a thick cuticle, legs were crushed and left to lyse overnight. Four samples had been stored in RNAlater^{TM} stabilizing solution (Invitrogen) and extraction followed the same protocol as the other samples except for an additional RNase step to remove preserved RNA.

Table 1. Specimens table. mtDNA = Numbers of individuals used in Φ_{ST} pairwise analyses and estimations for haplotype, nucleotide, and theta diversity. nuDNA= Number of individuals used in R_{ST} pairwise comparisons and Bayesian clustering analyses.

M. n. nigrolineatum	males		females		Total	
Site	mt DNA	nu DNA	mt DNA	nu DNA	mt DNA	nu DNA
Waiāhole	2	6	0	0	**2**	**6**
Poamoho	3*	3	0	1	**3**	**4**
Mānoa	6	8	3	3	**9**	**11**
Maʻakua	0	4	0	0	**0**	**4**
Kaipapau	10*	13*	1	1	**11**	**14**
Total	21	34	4	5	**25**	**39**
M. vagabundum	males		females		Total	
Site	mt DNA	nu DNA	mt DNA	nu DNA	mt DNA	nu DNA
Wailua River	18	29	1	4	**19**	**33**
Makaleha	17	23	0	2	**17**	**25**
Nā Pali	5	3	0	0	**5**	**3**
Kawaikoi	3*	3	0	0	**3**	**3**
Total	43	58	1	6	**44**	**64**

*1 individual had unknown sex and was grouped with males

Mitochondrial COII Sequencing The mitochondrial cytochrome oxidase subunit II (COII) gene was amplified in all individuals using the polymerase chain reaction (PCR). COII was amplified with primers C2-J-3102 (5'-aaa tgg caa cat gag cac aay t-3') and TK-N-3773 (5'-gag acc agt act tgc ttt cag tca tc-3') modified by Jordan *et al.* (2003). Approximately 650 base pairs were recovered per individual. PCR was carried out in 25 uL reactions containing Bioline Taq polymerase, <5 ng/uL genomic DNA, 0.4 uM of each primer, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, under the following conditions: 95°C/10 min followed by 35 cycles of [95°C/30 s, 50°C/30 s, 72°C/60 s] followed by a final extension of 72°C/10 min. PCR products were bidirectionally sequenced using Applied Biosystems BigDye terminator chemistry on an ABI 3730XL sequencer following the manufacturer's protocols. Sequencing was conducted at the University of Hawai'i at Mānoa's Advanced Studies of Genomics, Proteomics, and Bioinformatics facility. Each sequence was manually edited using Geneious v10. Ambiguous and short sequences were excluded. This process resulted in 24 COII sequence reads for *M.n.n.* representing 4 of our 5 sampling sites and 43 COII sequence reads for *M.v*. The COII dataset was supplemented with one additional *M.n.n.* individual collected from Poamoho from GenBank (AY179136) and one *M.v.* individual (AY179151) collected from Kawaikoi (Jordan *et al*., 2003).

Microsatellite Genotyping All individuals were genotyped for nuclear DNA variation. Individuals of *M.n.n.* were genotyped at 4 microsatellite loci while individuals of *M.v*. were genotyped at 3 microsatellite loci using primers developed by Jones *et al.* (2009) for closely related species (Table 2). PCR annealing temperatures were optimized for each primer pair with a gradient PCR (annealing temperature range: 50-60°C) and PCR reactions were carried out in 10 ul reactions containing Amplitaq DNA polymerase, <50 ng/uL genomic DNA, 0.2 uM of each primer, 0.2 mM dntp, and 3 mM $MgCl_2$. Thermocycling consisted of an initial denaturation at 95°C/3 min followed by 35 cycles of [95°C/30 s, locus-specific annealing temperatures (see Table 2) for 90 s, and 72°C/60 s], with a final extension at 72°C/10 min. The fragment analysis was conducted with an ABI 3730XL sequencer (Applied Biosystems) using the Liz size standard and visualized and manually genotyped using Geneious v10. Alleles that could not be identified reliably were reran or scored as missing data.

Table 2. Primer sequences of microsatellite loci used in both *M.n.n.* and *M.v.*

Species	Locus	Primer Sequence (5'-3')	Anneal Temp	Reference
M.n.n.	MegXa3: F	VIC-AGGAAGAACCCCTTTATCAC	54°C	Jones et al. 2009
	MegXa3: R	TTTCATTCAATAATTGGTTTTC		
M.n.n. & *M.v.*	MegXa5: F	6FAM-GTCATCGGTGAAAGATGAAT	55°C	Jones et al. 2009
	MegXa5: R	CCTTCCATCGATTTACCC		
M.v.	MegXa8: F	6FAM-CATCGCGCGAACAAAGGAAA	60°C	This paper
	MegXa8: R	ACGAGATTTGACGACCGAGTC		
M.n.n.	MegXa10: F	6FAM-TATATGTCCGTCCTCTCGAT	58.5°C	Jones et al. 2009
	MegXa10: R	GTTCTTAACGAACGCTGTTT		
M.n.n. & *M.v.*	MegEu12: F	6FAM-TCTCTGTCTCCCATCAATTC	56.5°C	Jones et al. 2009
	MegEu12: R	CCTTTCCCCTTGATAGAACT		

Analyses

As the analytic methods assume random mating, the microsatellite data were checked for deviations from Hardy-Weinberg expectations. In addition, recently there has been raised a concern about null alleles (Van Oosterhout *et al.*, 2004), in which an individual may in fact be a heterozygote at a locus, but for various reasons one allele fails to amplify (e.g., perhaps there is a problem with the primer binding site). The microsatellite data were also checked for null alleles and typographic errors using MicroChecker (version 2.2.3; Van Oosterhout *et al.*, 2004).

Genetic diversity Genetic indices of population diversity for the nuclear dataset were calculated with Arlequin ver 3.1 (Excoffier *et al.*, 2005) for each population. These included: number of alleles (Na), the observed heterozygosity (Ho) and expected heterozygosity (He). Deviations from Hardy-Weinberg proportions for each locus in each population were performed using the Markov chain exact test (Guo and Thompson, 1992) to estimate exact P-values, as implemented in Arlequin. Statistical significance was assessed with a sequential Bonferroni correction.

Genetic diversity indices were also calculated for the mitochondrial data. Sequences were evaluated at a per-site scale and globally across the entire COII locus. Haplotype diversity (the probability that 2 haplotypes drawn randomly from a population are different) and nucleotide diversity (mean number of pairwise differences per site between 2 sequences) within COII were calculated using Arlequin and were compared to congeners collected in the 1990s which had been evaluated with these same primers (Jordan *et al*., 2007). A haplotype network for COII was computed and visualized for each species using the package pegas (Paradis, 2010) in the R statistical computing environment (R Core Team, 2017). An additional *M. vagabundum* individual from Koaie stream in Waimea canyon was added solely to this haplotype network dataset (accessed from GenBank AY179152).

Assessing Population Structure Population structure was tested in both mitochondrial and nuclear datasets in three ways: first, pairwise genetic differentiation indices (F_{ST} analogues, Φ_{ST} and $R_{ST)}$ were estimated to assess the magnitude of differentiation among sample sites. Then an isolation-by-distance analysis was performed to test for correlation between genetic distances and geographic distances. Finally, a Bayesian clustering analysis was performed. MtDNA and nuDNA were assessed separately in all of these tests aside from the Bayesian clustering analysis where they were jointly combined.

Pairwise values of Φ_{ST} (an F_{ST} analogue that describes differences in sequence divergences rather than allele frequencies), were calculated for COII data while pairwise R_{ST} values (an F_{ST} analogue which assumes a step-wise mutation model rather than an infinite allele model; Slatkin, 1995) were calculated for microsatellite data using Arlequin. Statistical significance was assessed using 1000 bootstrap permutations and significance level was corrected using a Holm-Bonferroni correction.

Isolation-by-distance was tested using a Mantel test (5000 permutations) through Arlequin. The genetic differentiation measures Φ_{ST} and R_{ST} were measured against the shortest straight-line distance (km) between stream site global positioning system waypoints, calculated through Google Earth.

Microsatellite and mitochondrial data were also jointly analyzed for population clustering using STRUCTURE (version 2.3.4; Pritchard *et al.*, 2000, Falush *et al.*, 2003; burn-in = 25,000 followed by 100,000 Markov Chain Monte Carlo steps). Twenty iterations were run to test for varying numbers of population clusters (K) in the data, with hypothetical K-values ranging from 1 to 10. The admixture model was used, using default model parameters (allele frequencies correlated between populations and with no prior information on population origin). The most likely number of clusters was determined with the Evanno method (Evanno et al. 2005) as implemented in STRUCTURE HARVESTER (version 0.6.94; Earl and vonHoldt, 2012; http://taylor0.biology.ucla.edu/structureHarvester/). Individuals were assigned to clusters (individuals whose genotypes indicated that they were admixed were assigned to two or more clusters, accordingly). Results from STRUCTURE HARVESTER were visualized using the default settings in the software program CLUMPP (version 1.1.2; Jakobsson and Rosenberg, 2007) and software program distruct (version 1.1; Rosenberg, 2004).

Demographic analyses Demographic expansions and bottlenecks were tested for by comparing the sum of square deviations to a mismatch distribution model and by computing Tajima's D and Fu's F statistics in both the subpopulations and island-wide as implemented in Arlequin. Population bottlenecks and expansion were not tested for in the nuclear data as there was not enough microsatellite loci to provide sufficient statistical power.

Theta diversity was calculated per site and per species in Arlequin and used to calculate long-term effective population sizes by rearranging the equation $\theta=N_e u$ for haploid populations with inheritance only through the females. A mutation rate (u) of 2.69% My^{-1} was used based on the insect mitochondrial genes cytochrome oxidase subunit I and 16S RNA coding region mutation rates (Papadopoulou *et al*., 2010). Estimates within each population were calculated

along with the overall *Ne* as these *Ne* estimates should be less biased from population structure (Holleley *et al*., 2014).

Results

Genetic Diversity

Haplotype diversity was high in both species, 0.82 +/- 0.05 in *M.n.n.* and 0.67 +/- 0.05 in *M.v.*. Nucleotide diversity was also high at 0.0057 +/- 0.003 in *M.n.n*. and 0.0081 +/- 0.004 in *M.v.*. COII haplotype networks were visualized for both species (Figures 2,3). *M.n.n.* had a lot of haplotypes that were only found in a single population, only 3 of the 9 haplotypes were shared between populations. *M.v.* only had a couple of haplotypes that were unique to a specific population and had a haplotype network resembling a starburst pattern, except for one of the branches which had a lot of nucleotide differences. All of this is unlikely to be the result of a past bottleneck (see below in Demography).

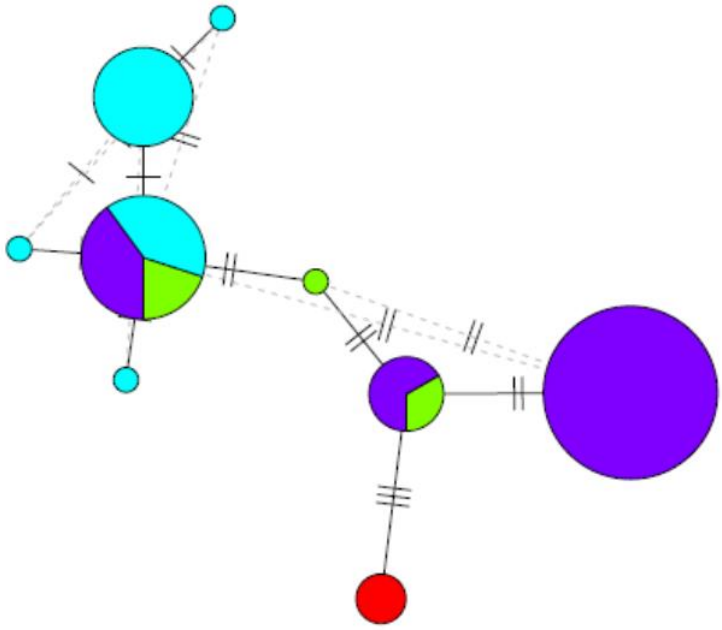

Figure 2. Haplotype network of COII in *M.n.nigrolineatum* representing 9 haplotypes from 25 different individuals. Circle size is relative to the frequency of haplotype copies. Branches represent a single nucleotide change while black lines on branches represent additional nucleotide changes. Dashed branches represent alternative connections. Haplotype copies are color coded by population: Purple is Kaipapau, red is Waiāhole, green is Poamoho, and light blue is Mānoa.

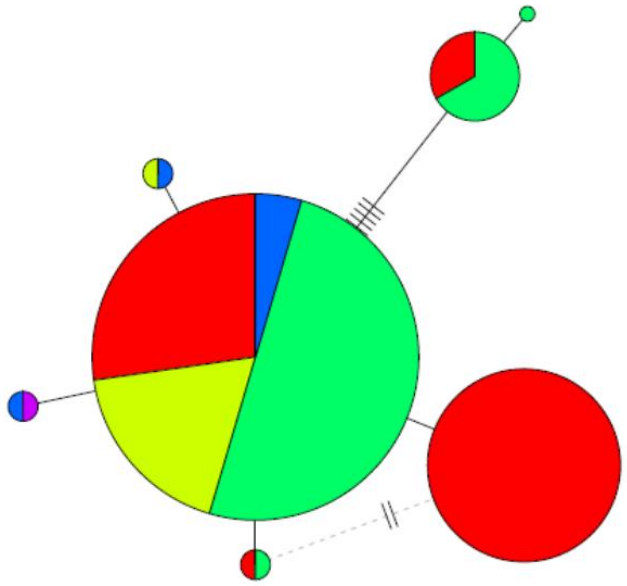

Figure 3. Haplotype network of COII in *M.vagabundum* representing 7 haplotypes from 45 different individuals. Circle size and branches represent the same thing as in Figure 2. Haplotype copies are color coded by population: Red is Wailua R N Fork, green-blue is Makaleha, green-yellow is Nā Pali, dark blue is Kawaikoi, and purple is Koaie stream in Waimea.

The long-term effective population size based on mean theta across all populations was 57.6 for *M.v.* and 84.8 for *M.n.n.* in the mitochondria assuming an equal sex ratio. Assuming this is only ¼ of the *Ne* based on nuDNA, long-term effective population sizes are closer to 230 in *M.v.* and 340 in *M.n.n..* Theta diversity did not differ much by subpopulation in *M.v.*, however it is very low in *M.n.n.*'s Kaipapau population, possibly due to smaller census population size at this location (Table 3).

Table 3. Theta diversity and effective population sizes from mitochondrial COII genes looking at populations with sample sizes ≥5 in *M.n.n*. and *M.v.*

Species	Sites	N	Theta	mt*Ne*
M.n.n.	Mānoa	9	1.47 ± 0.91	54.6
M.n.n.	Kaipapau	11	0.68 ± 0.52	25.2
M.v.	Nā Pali	5	1.44 ± 1.02	53.6
M.v.	Makaleha	17	1.77 ± 0.92	65.8
M.v.	Wailua R N Fork	19	2.00 ± 0.98	37.2

Theta and standard deviations calculated using number of variable sites along entire sequence length (S) using Arlequin. Ne was a rough estimate calculated using the equation Ne=theta/mu.

Of the 7 presumptive microsatellite loci, 2 were monomorphic in *M.n.n.* (MegXa10, MegXa5) and one was monomorphic in *M.v.* (MegXa8). MICROCHECKER detected the possibility of null alleles in all of the remaining loci but all polymorphic loci were retained in the analysis. Hardy-Weinberg proportions (HWP) only deviated significantly from Hardy-Weinberg equilibrium expectations in *M.v.* at MegEu12 in Wailua R N Fork (Table 4) although MegXa3 in *M.n.n*. and MegXa5 in *M.v.* had consistently low P values.

Table 4. Variation in microsatellite loci of 5 *M.n.n*. populations and 4 *M.v.* populations showing only polymorphic loci.

	MegXa3					MegEu12			
Sites	N	Na	He	Ho	P	Na	He	Ho	P
Mānoa	11	3	0.71	0.17	0.01	2	0.27	0.30	1
Waiāhole	6	4	0.77	0.17	0.01	2	0.17	0.17	1
Poamoho	4	3	0.60	0.33	0.21	2	0.25	0.25	1
Ma‘akua	4	2	0.53	0.00	0.20	2	0.33	0.33	1
Kaipapau	14	5	0.74	0.30	0.02	2	0.38	0.40	1
	MegXa5					MegEu12			
Wailua R N Fork									
	33	3	0.49	0.30	0.01	11	0.84	0.67	**0.002**
Makaleha	25	3	0.48	0.35	0.02	12	0.84	0.91	0.09
Kawaikoi	3	2	0.67	0.00	0.33	3	0.60	0.67	1
Nā Pali	3	1	0.00	0.00	1	3	0.77	0.67	1

Na = number of alleles; He = expected heterozygosity; Ho = observed heterozygosity; p = P-value for deviations from Hardy-Weinberg proportions; statistically significant P-values are in bold (sequential Bonferroni corrected).

Population Differentiation

M.n.n. showed significant population differentiation in the mitochondrial dataset between Kaipapau-Mānoa (Φ_{ST} = 0.370; Table 5) whereas the microsatellites showed no population differentiation (all R_{ST} were negative). Despite Kaipapau and Mānoa being the furthest sites away from one another, there is no evidence for strong straight-line isolation-by-distance in the mitochondria (r = -0.4, P = 0.80) and a positive but non-significant correlation between the microsatellite genetic difference and geographic distance (r = 0.5787, P = 0.08).

In contrast, *M.v.* showed significant population differentiation in the mitochondria between all populations (Table 6). Unlike *M.n.n.*, the microsatellite R_{ST}s were mostly positive, but none were significant. Similar to *M.n.n*., IBD was not supported for COII in *M.v.* (P = 0.12) nor between microsatellite genetic differences (R_{ST}) and geographic distance despite having a strong positive correlation (r = 0.869, P = 0.08). All of the resolution in population structure was therefore only statistically supported in the mitochondria for both species.

Table 5. Pairwise genetic differentiation index (mtDNA Φ_{ST}/nuDNA R_{ST}) values between populations of *M.n.n.* in O‘ahu.

Sites	Mānoa	Waiāhole	Poamoho
Waiāhole	0.371 / -0.037		
Poamoho	0.125 / -0.090	0.368 / -0.090	
Kaipapau	**0.369** / -0.025	0.605 / -0.058	0.373 / -0.078

Values in bold means populations are significantly differentiated (sequential Bonferroni corrected P-value < 0.00005)

Table 6. Pairwise genetic differentiation index (mtDNA Φ_{ST}/nuDNA R_{ST}) values between populations of *M.v.* in Kaua‘i.

Sites	Waialua N Fork	Nā Pali	Makaleha
Nā Pali	**0.438** / 0.085		
Makaleha	**0.458** / 0.037	**0.571** / 0.227	
Kawaikoi	**0.355** / 0.105	**0.499** / -0.196	**0.507** / 0.248

Values in bold means populations are significantly differentiated (sequential Bonferroni corrected).

Structure clustering results revealed panmixia across all populations. Assessment of the most likely number of hypothetical ancestral clusters (K-values) for 40 damselflies (*M.n.n.*) from 5 localities was K = 2 using the delta-K analysis. However, the delta-K value was extremely low (2.5) and the Evanno method does not perform well for when K=1 because it is unable to select this value of K (Figure 4). The results at K=2 did not show any meaningful population structure (Figure 5) as might be expected for a delta-K value equaling only 2.5. The same issue occurred in *M.v.* where the delta-K results revealed K=8 as the most likely number of population clusters even though delta-K was again extremely low (Figure 6). Ignoring the Evanno method, the highest L(K) was K=1 for both species and this value made the most sense when comparing the different Structure plots (Figures 5, 7).

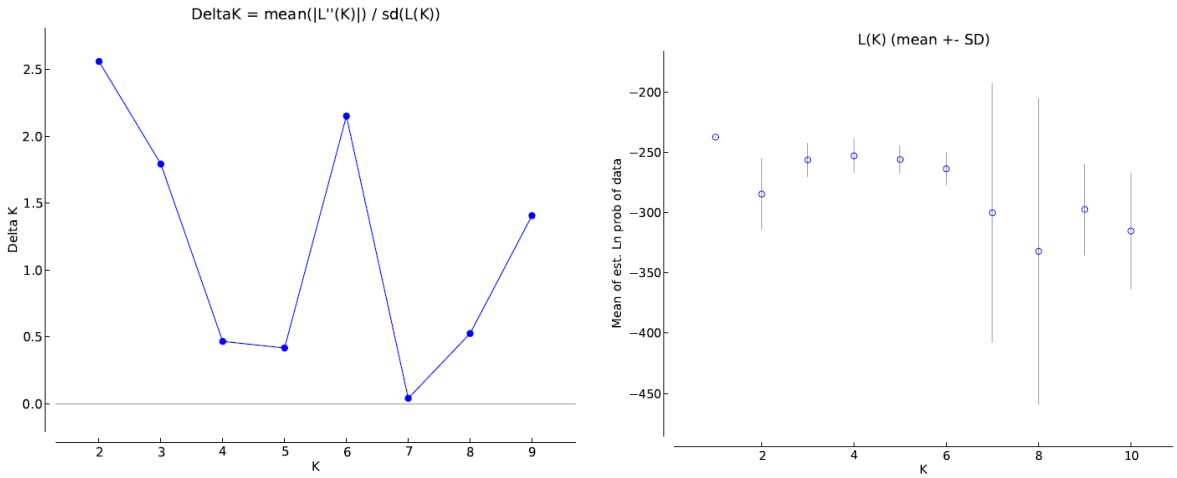

Figure 4. *M. n. nigrolineatum* most likely number of K clusters comparing delta K method to estimated mean likelihood K. The delta K method reveals a K=2 but with an extremely low delta K value (typically studies with population structure have delta K's in the 100s). The mean likelihood value revealed a K=1.

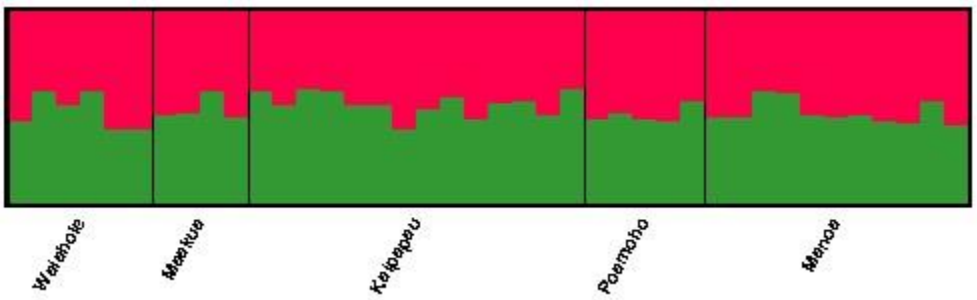

Figure 5. Structure bar plot for K=2 clusters for 40 damselflies (*M. n. nigrolineatum*) from 5 localities. Each vertical bar represents 1 individual, and its proportional ancestry from as many as 2 clusters which are shown in different colors. Black lines separate damselflies sampled in different streams.

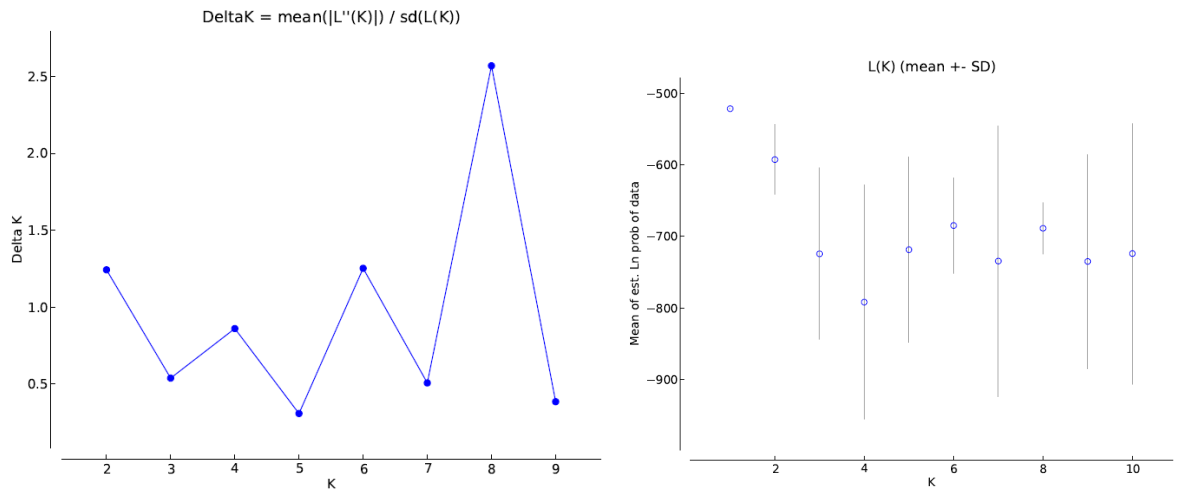

Figure 6. *M. vagabundum* most likely number of K clusters comparing delta K method to estimated mean likelihood K. The delta K method reveals a K=8 but with an extremely low delta K value (typically studies with population structure have delta K's in the 100s). The mean likelihood value reveals a K=1.

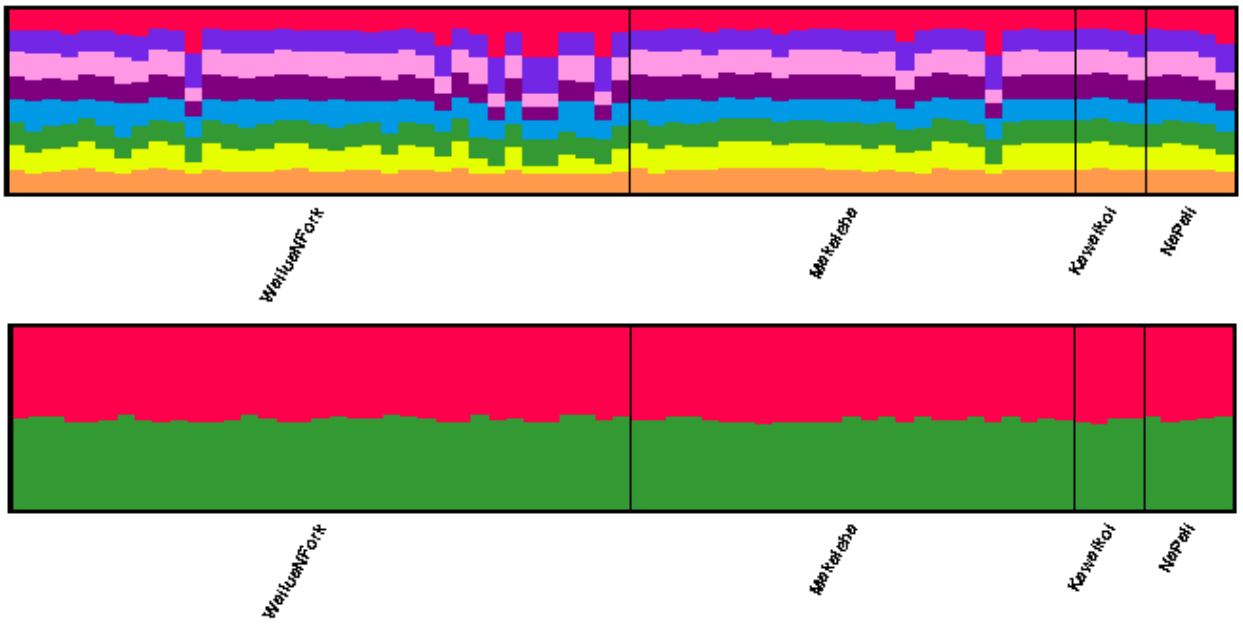

Figure 7. Structure bar plot for K=8 and K=2 clusters for 69 damselflies (*M. vagabundum*) from 4 localities. Each vertical bar represents 1 individual, and its proportional ancestry from as many as 2-8 clusters which are shown in different colors. Black lines separate damselflies sampled in different streams.

Demography

A mismatch distribution analysis of all haplotypes in *M.n.n.* exhibited a bimodal distribution but had non-significant SSD and Hrag values consistent with a possible recent population expansion (Figure 8). However, nonsignificant positive values of both Tajima's D (0.04, P = 0.57) and Fu's Fs (0.85, P = 0.7) suggest that there were no recent population expansions or bottlenecks. Subpopulations with sample sizes greater than 5 were independently assessed for population expansions or bottlenecks with similar results (Table 7).

All haplotypes within *M.v.* were very similar, generally within 1-2 nucleotide changes away from each other with a common haplotype in the center (Figure 3), generally a haplotype network portraying this "star-like" pattern implies a population expansion (Page and Holmes, 1998). A mismatch analysis of all haplotypes in *M.v.* was consistent with the haplotype network in that a population expansion fit with the observed data (Figure 9). Tajima's D was nonsignificant and negative (-0.30, P = 0.42) while Fu's Fs was nonsignificant and positive [5.18, P = 0.967; significance cut-off = 0.02 (Fu, 1997)]. When subpopulations were assessed independently, neutrality tests revealed no significant population expansions or bottlenecks. The closest situation to a recent bottleneck occurs in Makaleha (Tajima's D = 1.65; P = 0.048), however, this result is not significant after Bonferroni correction for multiple tests.

Table 7. Inferring population expansions or bottlenecks from gene diversity in distinctive subpopulations and in the species as a whole using COII data. Graphs of mismatch distribution by subpopulation are in the supplements

Sites	SSD p-value	Hrag p-value	Tajima's D	Tajima's D p-value
Mānoa	0.26	0.58	-0.843	0.258
Kaipapau	0.00	0.94	-0.127	0.39
Total *M.n.n.*	0.28	0.55	0.044	0.57
Nā Pali	0.04	0.52	-1.05	0.15
Makaleha	0.03	0.27	1.649	0.952
Wailua R N Fork	0.51	0.66	-0.648	0.286
Total *M.v*.	0.3	0.47	-0.3	0.41

Sum of deviations (SSD) P-value measures fit to a mismatch distribution model. P-values < 0.05 have a poor fit to a recent population expansion model. Raggedness (Hrag) p-values describe variation around the curve (less significant = better fit to population expansion curve). Table also includes results for a neutrality test (Tajima's D index and p-values). Significantly negative D is indicative of a population expansion, significantly positive D indicates a recent population bottleneck.

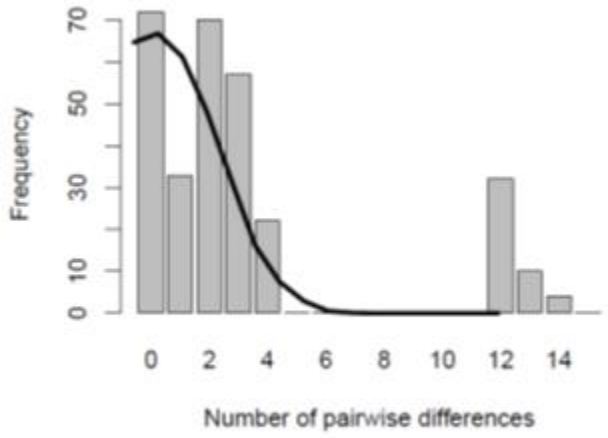

Figure 8. Mismatch distribution analysis of 25 *M. n. nigrolineatum* at the COII locus plotting observed frequencies of nucleotide differences in bars compared to simulations of a mismatch distribution model as a solid line.

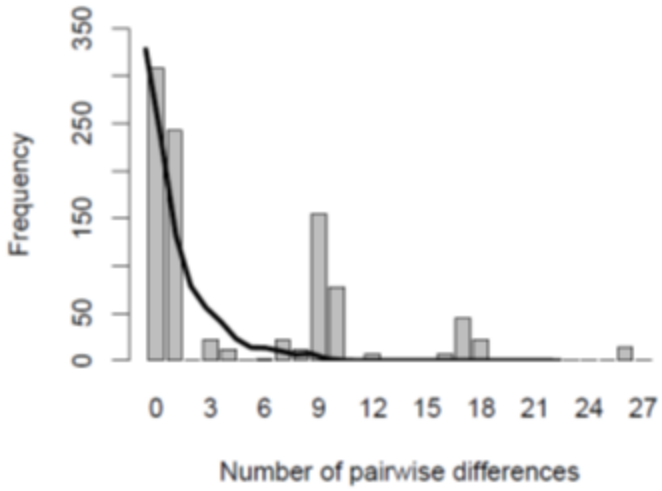

Figure 9. Mismatch distribution analysis of 44 *M. vagabundum* at the COII locus plotting observed frequencies of nucleotide differences in bars compared to simulations under a mismatch distribution model as a solid line.

Discussion

Genetic diversity within M.n.n. and M.v.

Both *M. n. nigrolineatum* and *M. vagabundum* have relatively high genetic diversity, likely as a result of genetic structuring within their respective islands. Demographic analyses show that the variation found in COII was the result of dispersal-mutation equilibrium and was not due to selection or recent expansions or bottlenecks. *M. vagabundum* is much more homogenous, many of the haplotypes found within one subpopulation could also be found in the other sample sites just at differing frequencies whereas each subpopulation of *M. n. nigrolineatum* harbored unique haplotypes that were not found in any of the other valleys. All of this is evidence that gene flow is limited in both species, but particularly in *M. n. nigrolineatum*.

The genetic diversity of *M. vagabundum* was consistent between the sampled populations. All of the sites were in protected areas around Kauaʻi and were located either deep in the interior of Kauaʻi or along the northwest Nā Pali coast which is also undeveloped and protected land. The genetic diversity within *M. n. nigrolineatum* differed more between populations and habitats were more variable in human impact. Poamoho was the most pristine site, but there were not enough individuals sampled to assess the genetic diversity at this site with confidence (however, given that of the 3 individuals sequenced from there, each had a different haplotype, the limited data suggests high diversity). The two sites that had adequate sample numbers were each severely impacted in different ways and had differing levels of genetic diversity. ʻAihualama stream in Mānoa has almost no native wildlife (with the exception

of *M.n.n.*) and is in one of the most biologically-poor watersheds in the state (Hawai‘i Division of Aquatic Resources), but genetic diversity was extremely high and there was an abundant population of *M. n. nigrolineatum* despite sharing the stream with introduced fish, amphibians, and invertebrates. Kaipapau was one of the poorest localities in terms of genetic diversity. An explanation for this might be stream diversions for agriculture resulting in ephemeral pools in what was once a perennial stream and reduced population sizes. Kaipapau also represents the edge of the species' range, however the effect of this might not be as strong if populations are rarely exchanging individuals across valleys.

Compared to most of the *Megalagrion* species that have been studied, haplotype diversity was intermediate in *M. n. nigrolineatum* and low in *M. vagabundum* whereas nucleotide diversity was low in *M. n. nigrolineatum* compared to other *Megalagrion* (Table 8). However, *M. n. nigrolineatum* and *M. vagabundum* are endemic to a single island whereas the other species studied have representatives on multiple islands and haplotypes were rarely shared across islands (Jordan *et al.*, 2007). The relatively low haplotype diversity in *M. vagabundum* may be due to having more gene flow between populations compared to *M. n. nigrolineatum* and compared to species with populations separated by ocean channels.

There has not been population genetics work done on other *Megalagrion* on Kaua‘i, so how *M. vagabundum* compares to other Kaua‘i populations is unknown. Notably, however, *M. n. nigrolineatum* has much higher genetic diversity than the other species populations studied on O‘ahu. *M. pacificum* was extirpated completely from O‘ahu, *M. xanthomelas* is only found at a single location on O‘ahu and is fixed at 1 haplotype (n=20), *M. calliphya* was never historically found on O‘ahu, and *M. hawaiiense* had lower haplotype diversity and nucleotide diversity in both Ka‘ala (n=7) and the Ko‘olau (n=6) than *M. n. nigrolineatum*. O‘ahu is highly impacted by habitat degradation and habitat destruction, but *M. n. nigrolineatum* seems to be doing much better than its sympatric congeners which means it has a higher likelihood of meeting the challenges of a changing environment assuming genetic diversity is not lost (Saccheri *et al.,* 1998; Reed and Frankham, 2003).

Table 8. Gene (h) and nucleotide diversity (π) of COII in *Megalagrion* calculated across entire species

Species	N	K	S	h	π	source
M. calliphya	40	20	27	0.95 ± 0.02	0.0085 ± 0.005	Jordan et al. 2007
M. hawaiiense	41	13	26	0.90 ± 0.02	0.0105 ± 0.006	Jordan et al. 2007
M. pacificum	19	6	32	0.60 ± 0.12	0.0011 ± 0.0009	Jordan et al. 2007
M. xanthomelas	91	16	32	0.82 ± 0.03	0.0116 ± 0.006	Jordan et al. 2007
M. n. nigrolineatum	25	9	7	0.82 ± 0.05	0.0057 ± 0.003	This study
M. vagabundum	44	6	10	0.67 ± 0.05	0.0081 ± 0.004	This study

N is sample size; K is number of different haplotypes; S is number of variable sites; h is average haplotype diversity and standard deviation; π is the average nucleotide diversity and standard deviation

Comparing genetic structure in M.n.n. and M.v.

Genetic analyses were motivated by an effort to understand population connectivity in a habitat generalist and a habitat specialist. Surprisingly, there was fine-scale spatial structure detected in the mitochondria in *M. vagabundum* which has a wide range of habitat preferences. Previous work has suggested that gene flow within an island is likely high in *Megalagrion* (Jordan *et al.,* 2005; Jordan *et al.*, 2007). However, even within these studies there is some evidence for demographically isolated units among populations within an island. For instance, *M. hawaiiense* has unique haplotypes at Onomea stream on Hawai'i (Jordan *et al.*, 2007). The present study supports the idea that other restrictions and barriers besides ocean channels play an important role in genetic structuring for this group.

M. n. nigrolineatum did not show statistically strong population differentiation between each of the sample sites. However, there is evidence for genetic structure in this species based on the unique haplotypes within subpopulations and high pairwise Φ_{ST} values. A post-hoc power analysis revealed that a lack of structure may have been an artifact of small sample sizes compared to *M. vagabundum* (Figure 10). If a lack of statistical power is to blame for the patterns seen, it is likely that even more populations are differentiated than demonstrated in this study. The possibility that each valley could harbor its own unique subpopulation should not be ruled out and seems to be supported between populations with sample sizes greater than n=5. This hypothesis requires further investigation. Adding hypothetical data to the

M. n. nigrolineatum dataset to try and bring up the power to match that in *M. vagabundum* revealed that adding 2 more microsatellite loci and 15 more individuals (5 from each of the smallest subpopulations) would provide enough resolution to detect structure in *M. n. nigrolineatum* (For more details see Appendix).

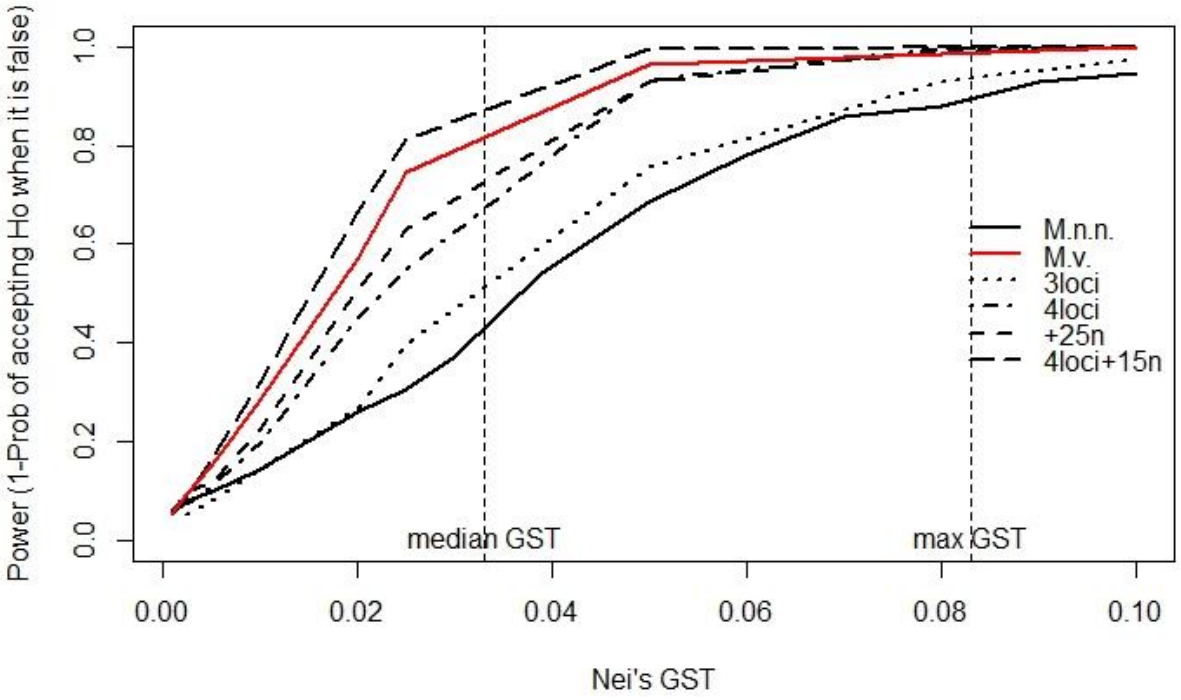

Figure 10. Power analysis of a Fisher's Exact Test comparing pairwise G_{ST} in *M. n. nigrolineatum* (black) to *M. vagabundum* (red). Dashed lines represent hypothetical changes to the *M. n. nigrolineatum* dataset to try to increase statistical power. Median and maximum pairwise G_{ST} were also calculated for *M. n. nigrolineatum* to see how observed G_{ST} compares to ability to detect statistical significance at that observed G_{ST}.

Likewise, lack of significant isolation-by-distance (IBD) and Bayesian population clustering in both species is also likely due to lack of power. Normally, the pattern of genetic differentiation among populations with distance provides more insight into processes affecting population structure (Miller *et al.*, 2002). If there is a lack of isolation-by-distance, this can be the result of recent colonization, physical barriers, genetic disequilibrium, long-distance dispersal, or historic changes in gene flow (e.g. Slatkin, 1993; Miller *et al*., 2002; Hughes *et al.*, 2003). Genetic differentiation and straight-line distance between sites were positively correlated for both species and markers with the one exception of *M. n. nigrolineatum* at COII which also had the smallest sample sizes. Adding more individuals from more than four populations would be

necessary to rule out IBD completely, but isolation-by-distance could be a powerful tool in the future to test for the effects of mountain ridges between valleys on genetic dispersal.

Despite large Φ_{ST} values, many of the pairwise R_{ST} values were below 0.03, and STRUCTURE has trouble detecting clusters at this fine scale (Latch *et al.*, 2006). Furthermore, most of the loci in the analysis were not in HWE. The lack of loci and the departure from model assumptions may have contributed to the program being unable to reject panmixia in either species.

Comparing mtDNA with nuDNA

It is difficult to assess whether the panmixia detected in the nuDNA is biologically meaningful having had only 2 loci to compare within each species. *M. vagabundum* at least seems to have enough power to detect structure in its nuDNA (based on the power analysis; Figure 10) yet populations remained undifferentiated. Finding more differentiation in the mtDNA than in the nuDNA is not rare or unusual, especially given its lower effective population size. If these two species are not well differentiated in the nuclear genome, this may rule out different populations being treated as separate evolutionary significant units (ESUs; Moritz 1994) although they may still be demographically distinct populations with great importance to the viability of the larger ESU.

Less differentiation in the nuDNA than in the mtDNA may sometimes suggest historic changes in gene flow or male-biased dispersal. However, these two explanations are unlikely in this system. To our knowledge, there has not been any recent changes to these habitats that would increase gene flow as might be suggested given the high mutation rates in microsatellites compared to the mitochondria. On the contrary, restriction and fragmentation of habitat (Polhemus, 1993; Englund, 1999) and introduction of potential invasive predators (Englund, 1999) would increase the risk of dispersal in these species. Most studies on damselfly dispersal find extremely high fidelity of adults to their breeding site (Banks and Thompson, 1985; Geenen *et al.*, 2000) and it is suggested that tenerals, or non-sexually mature emergent adults, are the primary dispersers. Tenerals spend the majority of their time away from breeding sites out foraging and females generally spend longer in the teneral stage than males do (Corbet, 1999). In most studies that took direct measures of damselfly dispersal between different sites, the authors found greater female dispersal (Beirinckx *et al.*, 2006). Preliminary results from a mark-recapture study on mature adult *M. n. nigrolineatum* also found evidence for female-biased dispersal whereas males had high site fidelity (Henry, in prep).

Factors influencing gene flow

Our results suggest that each study site is demographically isolated and that dispersal is therefore limited in both species, with stronger evidence supporting this claim in *M. vagabundum*. *M. vagabundum* is a relatively good flier and is not greatly restricted by ecological requirements so the cause of its limited dispersal is unknown. Elevational gradients and landscape heterogeneity has been shown to affect population structure in other damselfly species (Keller *et al.*, 2012) and although winged aquatic insects are expected to be able to cross into other lotic systems (Hughes, 2007; Hughes *et al.*, 2008) studies done on semi-aquatic insects in montane streams have found greater differentiation between water catchments than studies on semi-aquatic insects in lowland areas (e.g. Monaghan *et al.*, 2002; Elbrecht *et al.*, 2010).

Generally, damselflies are considered to be weak fliers only capable of dispersing up to 1 km (Conrad *et al.*, 2002) although there have been exceptions (Sato *et al.*, 2008). If *M. vagabundum* is just poor at flying further than 1 km from a breeding site, then there should be a distinct IBD pattern. As mentioned earlier, geographical distance was correlated with population differentiation in *M. vagabundum*. However, there was a negative association in mtDNA in *M. n. nigrolineatum*. The two populations that are leeward on the Koʻolau (Poamoho and Mānoa) had the lowest Φ_{ST} despite being quite far apart. A post-hoc AMOVA grouping these 2 populations together did not show great support for this ridgeline hierarchical structure (among group variation only accounted for 1.65% of the variation). This study was focused on range-wide structure comparing *M. vagabundum* to *M. n. nigrolineatum*, but an experimental design that tests the effects of the mountain ridges could better show whether these ridges have an impact on population connectivity and whether populations might be better grouped by valley or if individuals avoid all overland dispersal and are differentiated more by stream, or perhaps by larger ridges but not all ridges, *etc*.

Conservation implications

Habitat specificity is a well-known ecological attribute of species that increases the risk of extinction (Waldron *et al.*, 2000). One of the mechanisms for this is reduced population connectivity, leading to more isolated subpopulations with increased inbreeding and vulnerability to extinction (Frankham, 1995; Saccheri *et al.*, 1998; Frankham, 2005). This reduced population connectivity was not only suggested in *M. n. nigrolineatum*, the habitat specialist, but it was found in *M. vagabundum*, a habitat generalist. They both had high Φ_{ST} values indicating low dispersal between sites. Past and present water management practices have severely

degraded and altered streams where these damselflies are found, particularly on O‘ahu (Polhemus and Asquith, 1996). With increasing habitat loss, range shifts are less likely due to low dispersal and subpopulations are left either having to adapt or go extinct with the possibility of losing rare or unique alleles, particularly in *M. n. nigrolineatum* which harbored many more unique alleles within its subpopulations than *M. vagabundum*. In addition to habitat loss, modifications of rain seasons and increasing drying due to climate change (e.g. Deutsch *et al.*, 2008) could reduce stream flow even more and present additional challenges to these species.

Effective conservation strategies should take genetic factors into consideration (Frankham, 2005; Feindt *et al.*, 2014). Each valley constituted an important management unit. More markers and individuals may contribute a better understanding on the gene flow in *M. n. nigrolineatum*, though there was likely enough power in *M. vagabundum* to rule out differentiation within the nuclear genome. Long-term protection of *M. n. nigrolineatum* should focus on protecting streams where they are found. Comparing Mānoa to Kaipapau suggests that stream diversions have a larger impact on population numbers and genetic diversity than non-native introductions alone. Future augmentation or reintroduction might be helpful for extirpated populations of *M. n. nigrolineatum*, though there has not been much success with other *Megalagrion* (see Preston, 2013). Further assessment of the nuclear genome would also be needed to rule out any distinct recovery units.

APPENDICES

Appendix A. Example of Long-term Effective Population Size Calculation

$\theta = Neu$ in mitochondria

$Ne = \frac{\theta}{u}$

$u = 0.0269$ in insect mitochondria

Kaipapau: $\theta = 0.683$

$Ne = \frac{0.683}{0.0269} = 25.4$ based on mitochondrial DNA

$Ne = 4 * mtNe$

$Ne = 4 * 25.4 = 101.6$

Appendix B. Power Analysis

The post-hoc power analysis was done using the software POWSIM (Ryman and Palm, 2006) developed to assess statistical power when testing for population differentiation. The results of a Fisher's exact test were evaluated in the *M. vagabundum* dataset (2 microsatellite loci MegEu12 and MegXa5 at the observed allelic frequencies, along with sample number and sample sizes that were the same as when calculating R_{ST}) simulated at different values of Nei's G_{ST} ("M.v." in Figure 10). These were then compared to *M. n. nigrolineatum* with the unaltered dataset (2 microsatellite loci MegXa3 and MegEu12 at the observed allelic frequencies, sample number and sample sizes were the same as when calculating R_{ST}) simulated at different values of Nei's G_{ST} ("M.n.n." in Figure 10).

The *M. vagabundum* dataset had adequate power to detect population structure, however, *M. n. nigrolineatum* was lacking statistical power so different hypothetical alterations were done to the dataset to assess different strategies on how to increase power in *M. n. nigrolineatum.* A third microsatellite loci was added using the allelic frequencies found in MegXa5 in *M. vagabundum* ("3loci" in Figure 10), a fourth microsatellite loci was added using the allelic frequencies in both MegXa5 and MegEu12 in *M. vagabundum* ("4loci" in Figure 10), no microsatellite loci were added but 5 more individuals were sampled per population (for a total of 25 more individuals, nearly doubling n) ("+25n" in Figure 10), and finally adding the 2 microsatellite loci MegXa5 and MegEu12 along with 5-6 more individuals from the populations that had n<10 to bring those sample numbers up to n=10 (Poamoho, Waiāhole, Ma'akua) ("4loci+15n" in Figure 10).

Nei's pairwise G_{ST} was calculated for *M.n.n.* and *M.v.* using GenoDive v2.Ob27 (Meirmans and Van Tienderen, 2004) and maximum and median were calculated combining all pairwise G_{ST} results for both species in R software.

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