

Phylogeographic patterns of Hawaiian *Megalagrion* damselflies (Odonata: Coenagrionidae) correlate with Pleistocene island boundaries

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Abstract

The Pleistocene geological history of the Hawaiian Islands is becoming well understood. Numerous predictions about the influence of this history on the genetic diversity of Hawaiian organisms have been made, including the idea that changing sea levels would lead to the genetic differentiation of populations isolated on individual volcanoes during high sea stands. Here, we analyse DNA sequence data from two closely related, endemic Hawaiian damselfly species in order to test these predictions, and generate novel insights into the effects of Pleistocene glaciation and climate change on island organisms. *Megalagrion xanthomelas* and *Megalagrion pacificum* are currently restricted to five islands, including three islands of the Maui Nui super-island complex (Molokai, Lanai, and Maui) that were connected during periods of Pleistocene glaciation, and Hawaii island, which has never been subdivided. Maui Nui and Hawaii are effectively a controlled, natural experiment on the genetic effects of Pleistocene sea level change. We confirm well-defined morphological species boundaries using data from the nuclear EF-1 α gene and show that the species are reciprocally monophyletic. We perform phylogeographic analyses of 663 base pairs (bp) of cytochrome oxidase subunit II (COII) gene sequence data from 157 individuals representing 25 populations. Our results point to the importance of Pleistocene land bridges and historical island habitat availability in maintaining inter-island gene flow. We also propose that repeated bottlenecks on Maui Nui caused by sea level change and restricted habitat availability are likely responsible for low genetic diversity there. An island analogue to northern genetic purity and southern diversity is proposed, whereby islands with little suitable habitat exhibit genetic purity while islands with more exhibit genetic diversity.

Keywords: phylogeography, Pleistocene, sea level change

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Introduction

Enormous advances have been made in deciphering the evolutionary and demographic processes that shaped continental biotas during the Pleistocene (e.g. Taberlet *et al.* 1998; Hewitt 2000; Good & Sullivan 2001; Johnson 2002). In contrast, the effects of Pleistocene climate change on

island biogeography have received much less attention (but see Chown 1990; Ruedi *et al.* 1998; Buckley *et al.* 2001; Arensburger *et al.* 2004). This may be due to the lack of direct glacial effects on many of the world's biotically rich islands. This subject merits more attention, however, because glacially mediated sea level variation during the Pleistocene was on the order of 120 m (Matthews 1990) and declines in sea level dramatically altered the size and shape of islands. In the Indonesian archipelago, for example, over 1.53 million km² of new land was available to terrestrial organisms at maximum glaciation [~17 000 years ago (ka)] and details of the topography and river systems in this

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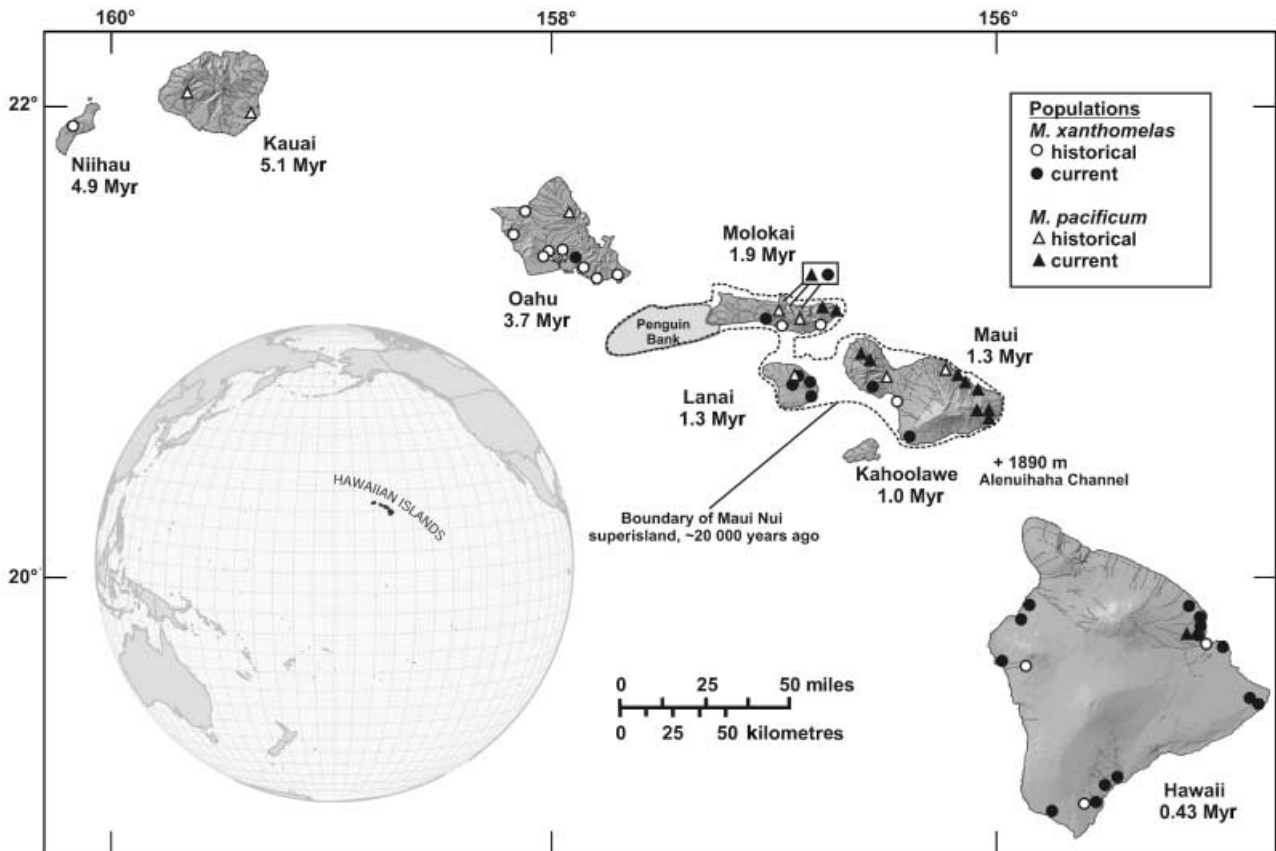


Fig. 1 Map of the high Hawaiian Islands, showing current and historical collection sites for *Megalagrion xanthomelas* and *Megalagrion pacificum*, and the current extent of the submarine Penguin Bank. Island ages based on K-Ar dating are shown (Clague & Dalrymple 1987). In periods of low sea stands, the islands of Molokai, Maui, Lanai, and Kahoolawe were connected into a super-island referred to as Maui Nui. Approximate boundaries of this island as it existed 20 ka are shown with a dashed line (after Price & Elliott-Fisk 2004). Note that at this time Kahoolawe stood alone.

now-flooded land are known (Voris 2000). Often, changes in island size and shape have had biological consequences (see Davies & Bermingham 2002 for an example involving Caribbean butterflies). These consequences are amplified by several properties of islands: their degree of isolation, their relatively small size compared to continents, and their preponderance of endemic species.

Episodes of warming and cooling during the Pleistocene occurred at regular intervals (Hewitt 2000) and led to periodic and at times rapid fluctuations in global sea level and thus island sizes. The genetic consequences of such changes on islands should differ from those proposed for northern continental systems (Hewitt 2000), where a pattern of 'southern (genetic) richness to northern purity' has been proposed (Hewitt 1996). Glaciation reduced available habitat for most studied northern continental taxa. When the glaciers disappeared, vast areas of open habitat became available (Hewitt 2001; Taberlet & Cheddadi 2002), although exceptions may exist (e.g. species that inhabit mountain tops or sky islands, see Masta 2000; Knowles 2001). On oceanic islands the situation may have been quite different,

with glaciation leading to lower sea levels and sometimes dramatic *increases* in total island area. In interglacial periods, as Northern Hemisphere continental organisms were increasing their ranges northward from refugia, many island taxa were losing available habitat and becoming more isolated on higher ground.

The islands of Hawaii present an excellent system for studying the effects of Pleistocene climate change on tropical oceanic islands, in large part because their geomorphological history is becoming known in some detail (Carson & Clague 1995; Clague 1996; Hotchkiss *et al.* 2000; Price & Clague 2002). The Hawaiian Islands are the product of a mantle plume hot spot that has existed for at least 80 million years (Myr). This hot spot has remained stationary as the Pacific tectonic plate has drifted to the north and northwest above it (Carson & Clague 1995), and lava has broken through Earth's crust regularly to form islands. The Hawaiian Islands are thus arranged linearly, and increase in age as one moves to the northwest (Fig. 1). Kauai is the oldest of the high Hawaiian Islands, at 5.1 Myr, while Hawaii is the youngest, at roughly 0.5 Myr. As

islands move further from the hot spot, subsidence and erosion decrease their height until they become atolls and ultimately underwater sea mounts. The depths of sea channels between Hawaiian Islands are variable, and some islands that are currently separate have been connected in the past.

In particular, the islands of Maui Nui (the ensemble of Molokai, Lanai, Maui, and Kahoolawe) rise from a large, shallow platform. These islands have been connected during more than 75% of the past 1.2 Myr and, excluding Kahoolawe, as recently as 15–20 ka (Price & Elliott-Fisk 2004; see Fig. 1). Molokai and Oahu were connected much earlier, before approximately 2 million years ago (Ma), via a high connection through the current Penguin Bank (Fig. 1) and a broad plain northwest of Molokai. Together these islands formed the 'Oahu Nui' super-island (Price & Elliott-Fisk 2004). Several authors have emphasized that understanding Pleistocene island connections such as these is fundamental to our understanding of the evolution of the Hawaiian biota (Funk & Wagner 1995; Craddock 2000). The island of Hawaii is currently separated from Maui by the 1890-m-deep Alenuihaha Channel and has never been physically connected to any other Hawaiian high island, even during low sea stands (Carson & Clague 1995).

Organisms on Maui Nui and Hawaii have effectively undergone a controlled natural experiment on the genetic effects of Pleistocene sea level change. At glacial maxima 20 ka, Maui Nui was roughly 5900 km² in size, or nearly twice its current size (Price & Elliott-Fisk 2004). During previous low sea stands, Maui Nui was even larger, and at its maximum extent 1.2 Ma, its ~14 000 km² exceeded the current size of the Big Island of Hawaii (10 458 km²). The contiguous Maui Nui included expansive tracts of lowland habitat (Price 2004), extensive lowland rivers (whose courses can be seen in sonar-generated seafloor maps), and large coastal pools (J. Price, personal communication). At glacial minima, such as the current high sea stand, Maui Nui is fragmented into four unconnected islands, while Hawaii remains contiguous.

Current patterns of genetic diversity on these islands are the product of at least three possible evolutionary scenarios. Under scenario 1, ocean channels between islands represented significant barriers to dispersal, and the land connections that existed between islands during periods of low sea stands did not facilitate gene flow. This idea has received much support in the literature by authors who have proposed that the formation of sea channels between the individual sub-islands of the Maui Nui complex may have led to greater isolation of single volcano populations (Funk & Wagner 1995; Roderick & Gillespie 1998; Craddock 2000). Under scenario 2, ocean channels between islands represented significant barriers to dispersal, but organisms freely colonized and moved across land connections that existed during low sea stands (Piano *et al.* 1997). Under scenario 3, ocean channels were not significant barriers to

dispersal and organisms moved freely between all land areas.

Predicted genetic patterns allow us to test these scenarios. Under scenario 1, we would expect each volcano to harbour populations with significant divergence due to interrupted gene flow. Under scenario 2, we would expect to see little to no genetic differentiation within land areas that are contiguous at low sea stands: Maui Nui and the island of Hawaii. Under scenario 3, we would expect to see signs of gene flow even between Maui Nui and Hawaii, in spite of the permanent ocean barrier between them.

The endemic Hawaiian damselflies *Megalagrion xanthomelas* and *Megalagrion pacificum* are widespread, freshwater insects that are part of a spectacular radiation of 23 species that occupy a wide variety of habitats (Jordan *et al.* 2003). These species offer an excellent opportunity to explore the phylogeographic impacts of Pleistocene climate change for several reasons. First, these taxa are sister species that are well-defined morphologically (Polhemus 1997). Second, they are geographically widespread, with current populations spanning five Hawaiian islands. Their distribution includes the islands of Maui Nui and Hawaii, making them ideal for testing the scenarios outlined above. Intensive surveying and sampling in the 1990s delineated the distribution of these species and generated a valuable collection of alcohol-preserved specimens (Polhemus 1996). Third, although they can exist in sympatry, these species prefer slightly different habitats, and so may react differently to sea level change. *Megalagrion xanthomelas* is generally most abundant at sea level and can develop large populations in spring-fed and even brackish coastal wetlands. *Megalagrion pacificum* is found in shady side channels of streams at low and intermediate altitudes (Polhemus & Asquith 1996).

Empirical studies examining each of the three scenarios outlined earlier are needed, and will complement the large number of evolutionary studies correlating Hawaiian biotic patterns with plate tectonics, volcanism, and ecology (see Wagner & Funk 1995 and references within). In this study, we apply phylogenetic and phylogeographic methods to these two Hawaiian damselfly species to examine the effects of Pleistocene climate change on them. We examine the geographical distribution of mitochondrial genetic variability across the five islands that make up the current range of these species to test the three possible scenarios outlined above. Finally, we explore the implications of our results for the Pleistocene phylogeography of island organisms in general.

Materials and methods

Sampling and specimens examined

Megalagrion xanthomelas is abundant on Hawaii and the north shore of Molokai, common on Lanai, and relictual on Maui and Oahu (Fig. 1). Although Perkins (1913) noted that

M. xanthomelas was once common in Honolulu gardens, it is now represented on Oahu by a small population found in a hundred metres of stream at the Tripler Army Medical Center (TAMC). A single specimen was collected in 1947 on Niihau, but the species has never been documented on Kauai. *Megalagrion pacificum* is most abundant on Molokai, where it often occurs near *M. xanthomelas*. It persists on Maui and a single individual was recently collected from Hawaii (Englund 1999), although no significant populations have been found there. *Megalagrion pacificum* was once found on Kauai, Oahu, and Lanai, but is now extirpated from these islands (Polhemus & Asquith 1996; Polhemus 1997).

We examined a total of 130 *M. xanthomelas* and 27 *M. pacificum* individuals (Table 1). The disparity in these numbers reflects the relative abundance of *M. xanthomelas* compared to the more localized *M. pacificum*. Our collections of these species were from 25 populations and five islands, effectively spanning their current known range, including rare *M. xanthomelas* individuals from remnant populations on Maui and Oahu. Efforts were made to analyse 20 individuals from each population or geographically close group of populations. However, in many cases this was not feasible due to variation in population sizes and the logistics of visiting many different localities. Wings, abdomens, heads, and terminalia of each specimen have been preserved in individual vials. These voucher specimens will be deposited at the Bernice P. Bishop Museum in Honolulu, and the Kilauea Field Station, Pacific Island Ecosystems Research Center, US Geological Survey, Hawaii Volcano National Park. Extracted DNA will remain in the care of S.J.

DNA extraction and sequencing

DNA was extracted from damselfly thoracic muscles using either a standard phenol–chloroform method (Sambrook *et al.* 1989) or a QIAGEN DNeasy Tissue Kit. Mitochondrial DNA (mtDNA) and nuclear primer sequences and conditions for polymerase chain reaction (PCR) and cycle sequencing are reported elsewhere (Jordan *et al.* 2003). We generated 663 bp of DNA sequence data from the mitochondrial COII gene, which we expected to show informative variation within species (Simon *et al.* 1994) using two primers: C2-J-3102 and TK-N-3773. In order to explore the possibility of introgressive hybridization between these species, we augmented a pre-existing nuclear DNA data set by sequencing approximately 1000 bp of the EF-1 α gene from two individuals using two primer pairs: EF1-F-2361 and EF1-R-2765 for the 5' end and EF1-F-2652 and EF1-R-3093 for the 3' end (Jordan *et al.* 2003). All sequence autapomorphies and nonsynonymous mutations were verified by rechecking the original chromatograms.

In order to identify possible contamination, extraordinary results were double checked by gathering the data again. The Hawaii island *M. pacificum* mtDNA was extracted,

amplified, and sequenced twice, in separate months. The mtDNA from two *M. xanthomelas* individuals from Kaloko, Hawaii island, that were found to bear haplotypes from another clade was amplified and sequenced twice, again in different months.

mtDNA sequence analysis

DNA sequences were aligned by eye and SEQUENCHER version 3.0 (GeneCodes). Basic sequence attributes, including raw (*p*) haplotype divergences, codon site-specific mutation rates, base composition bias, and amino acid substitutions were calculated using MEGA2 (Kumar *et al.* 2001) and PAUP* version 4.0b6–10 (Swofford 1999).

We used three separate classes of data analysis on the mtDNA data, explained in detail below, (i) phylogenetic analysis, (ii) tcs (Templeton, Crandall, Sing) parsimony and nested clade analysis (NCA: Templeton *et al.* 1992; Templeton 1998) and (iii) analysis of molecular variance (AMOVA: Excoffier *et al.* 1992).

Phylogenetic analysis

Maximum parsimony (MP) and maximum likelihood (ML) were implemented using PAUP* 4.0b6–10 (Swofford 1999). *M. xanthomelas* and *M. pacificum* display high genetic distances from all other *Megalagrion* species and initial data exploration suggested that these distant outgroups could lead to poorly resolved trees. We therefore carried out all MP, ML, and Bayesian analyses using no outgroups, and rooted the resulting trees a posteriori (see below).

We performed heuristic, equally weighted MP searches using 100 random-addition-sequence replicates and TBR (tree-bisection–reconnection) branch swapping. MP bootstraps were performed using a full heuristic search (10 random-addition-sequence replicates, TBR branch swapping) and 1000 pseudoreplicates.

Because appropriate ML models are important in molecular systematics (Sullivan & Swofford 1997), we selected the best model for our data using the method of Frati *et al.* (1997). We considered 16 different likelihood models and used a likelihood-ratio test to select the simplest model that was not significantly different from the best fitting model (GTR + I + Γ). This simplest model (HKY + I) was used in all ML analyses in order to minimize computing time and reduce the variance of the estimated parameters. Parameter values for this model were estimated on one of the MP trees, and fixed for the first ML heuristic search (10 random-addition-sequence replicates, TBR branch swapping, all best trees retained). After a full heuristic ML search was completed, we re-estimated model parameters on the resulting tree and used those parameters to search again, repeating this process until the new search recovered the same topology as the previous search. In ML bootstrap searching, model parameter values were

Table 2 Population pairwise F_{ST} 's calculated from the AMOVA procedure and 3024 permutations. Values that are significantly high at a 0.05 level are marked with an asterisk (*). Populations with small sample sizes are included for exploratory comparison

Population	N	1	2	3	4	5	6	7	8	9	10	11
1. Molokai <i>M. pacificum</i>	21											
2. Maui <i>M. pacificum</i>	5	0.52*										
3. Oahu	20	0.98*	0.97*									
4. N. Molokai	20	0.94*	0.89*	0.45*								
5. S. Molokai	2	0.96*	0.87*	1.00*	-0.05							
6. Lanai	10	0.94*	0.88*	0.38*	-0.02	0.17						
7. Maui	5	0.95*	0.87*	0.85*	0.26*	0.13	0.31*					
8. N. Hilo	19	0.92*	0.85*	0.93*	0.90*	0.88*	0.89*	0.88*				
9. S. Hilo	13	0.94*	0.89*	0.96*	0.93*	0.92*	0.92*	0.92*	0.02			
10. Kau	20	0.95*	0.92*	0.97*	0.94*	0.95*	0.94*	0.94*	0.06	0.08		
11. Kanonone	6	0.96*	0.90*	0.99*	0.94*	0.95*	0.94*	0.94*	0.04	-0.06	0.09	
12. Kaloko	16	0.84*	0.72*	0.84*	0.81*	0.71*	0.77*	0.75*	0.09*	0.07	0.09*	0.00

estimated from the full data set. We carried out 100 pseudoreplicates using heuristic searches with one random-addition-sequence replicate and retaining one tree per replicate, a time-saving measure shown to have no effect on bootstrap results (DeBry & Olmstead 2000).

For comparison, and as another estimate of nodal support, we carried out a Bayesian analysis (Larget & Simon 1999; Simon & Larget 2000; Lewis 2001) using the MRBAYES software package of Huelsenbeck & Ronquist (2001). We used an HKY + I model and ran four chains of 1 million steps each. Three of these chains were heated (temperature = 0.20), and all prior probabilities were uniform. Trees from a burn-in period of 10 000 generations were discarded before final clade posterior probabilities were calculated.

Rooting of the MP, ML, and Bayesian network was determined using the technique of Lundberg (1972) as described by Swofford *et al.* (1996) and an ML analysis under the GTR + I + Γ model using four outgroups (two sister taxa, *Megalagrion orobates* and *Megalagrion nigrohamatum*, and two taxa with the lowest genetic distances to the study species, *Megalagrion oahuense* and *Megalagrion adytum*). Under Lundberg rooting, an ancestral taxon is added to a branch of an unrooted network so that the overall tree length increases the least. This procedure was run four times, each time using one of the four outgroup taxa listed above to set the ancestral states.

tcs parsimony and nested clade analysis

Traditional phylogenetic methods are often not well suited to the analysis of recently diverged genetic data (Posada & Crandall 2001). In order to understand the relationships between closely related haplotypes, we implemented statistical or *tcs* parsimony (Templeton *et al.* 1992) using the *tcs* program (Clement *et al.* 2000). This method can be used to estimate the maximum number of substitutions

that can separate two closely related haplotypes before there is more than a 5% chance that a multiple hit has occurred. In order to explore correlations between haplotypes and geography and the processes that created them, we performed NCA (Templeton 1998) using the GEODIS software package (Posada *et al.* 2000).

We carried out NCA on both haplotype networks justified by *tcs* parsimony, with geographical locations specified by latitude and longitude. NCA consisted of two steps. First, an exact permutational contingency test (exact test) was used to identify significant geographical associations of haplotypes (Templeton & Sing 1993; Templeton *et al.* 1995). Only clades with a significant result for the exact test were examined further using data randomization and several metrics of geographical distribution. We explored the test results using the most recent inference key from the GEODIS website. This inference key summarizes patterns of haplotype distribution expected when genetic structure is the result of restricted gene flow, vicariant fragmentation events, or past range expansions.

AMOVA

In order to further understand the geographical patterns of genetic variation, we performed an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) using ARLEQUIN version 2.0 (Schneider *et al.* 2000) and 10 000 permutations. Uncorrected genetic distances were used in the AMOVA. Pairwise F_{ST} 's based on 3024 permutations were calculated between all populations, regardless of the number of individuals sampled. Haplotypes in the AMOVA were structured by the geographical locations (Table 2), which were in turn arranged into three groups: Oahu and Maui Nui *M. xanthomelas*, Maui Nui *M. pacificum*, and Hawaii Island haplotypes. Gene diversity (equivalent to expected heterozygosity of diploid data), the mean number of

pairwise differences between all haplotype pairs, nucleotide diversity, and theta (S) were calculated using ARLEQUIN for the three clades found by the phylogenetic analysis.

EF-1 α sequence analysis

In order to confirm species boundaries, a nuclear gene, EF-1 α , was sequenced from two *M. xanthomelas* individuals from the Kaloko population on Hawaii, which carried mtDNA common to Maui Nui. These sequences were combined with sequences from four *M. xanthomelas* (Oahu, Lanai, Molokai, S. Hilo), three *M. pacificum* (Molokai, Maui, Hawaii), and four outgroups (the same outgroup taxa as above) that were generated for a phylogenetic study of the entire genus (Jordan *et al.* 2003). The K2P + Γ model was deemed appropriate for the data using the method outlined above, and ML heuristic and bootstrap searches (200 pseudoreplicates) were carried out.

Results

The alignment of EF-1 α data, including outgroups, was 1039 bp long, with 54 parsimony-informative characters (GenBank Accession nos DQ083372–DQ083373). Phylogenetic analysis of these sequences strongly confirmed the morphological hypothesis of reciprocal monophyly between these species (results not shown). Monophyly of *Megalagrion xanthomelas* was supported with a bootstrap value of 96%, and monophyly of *Megalagrion pacificum*, including the single individual from Hawaii island, was supported with a bootstrap value of 85%.

We sequenced 663 bp of the COII gene from 157 *M. xanthomelas* and *M. pacificum* individuals and four outgroup species. Haplotype sequences are available from GenBank (Accession nos DQ083341–DQ083371) and aligned from SJ. We identified 31 unique ingroup haplotypes (Table 1). There are 37 variable sites in the ingroup, 23 of which are parsimony informative. The majority of variable sites, 26 (70%), are third codon position, 2 (5%) are second positions, and 9 (24%) are first positions. Eleven substitutions result in amino acid changes. Seven of these are between chemically similar residues. Overall the data show a strong AT bias (69.8%), and this bias varies by codon position (1st – 55%, 2nd – 66%, 3rd – 88%). Nucleotide bias does not vary among haplotypes (chi-squared $P = 1.0$).

Phylogenetic analysis

Maximum-parsimony, maximum-likelihood, and Bayesian methods all converged on the same mtDNA topology (Fig. 2) with three well-supported clades: (i) *M. xanthomelas* haplotypes predominantly from Oahu and Maui Nui (NorthX clade), (ii) *M. pacificum* haplotypes from Maui Nui

(NorthP clade), and (iii) *M. xanthomelas* and the single *M. pacificum* from Hawaii Island (Hawaii clade). Relationships within these clades are poorly resolved (Fig. 2). The average uncorrected genetic distance between the NorthX and NorthP clades is lower (1.7%) than the distance between either of them and the Hawaii clade (2.4% and 2.2%, respectively). Lundberg and ML rooting analyses unanimously supported the rooting indicated in Fig. 2. The resultant topology suggests that *M. xanthomelas* is paraphyletic with respect to *M. pacificum*.

There were several exceptions to the overall patterns of mtDNA haplotype distribution. First, two *M. xanthomelas* individuals from the Kaloko population on Hawaii bore NorthX haplotypes Xa and Xb, which were otherwise only found on Oahu and Maui Nui. Second, the individual *M. pacificum* from Hawaii bore haplotype Pch, which is more closely related to Hawaii *M. xanthomelas* than to Maui Nui *M. pacificum* (Fig. 2).

TCS parsimony and nested clade analysis

TCS parsimony analysis indicated that haplotypes differing by 11 or fewer substitutions could be combined into contiguous networks. This resulted in two haplotype networks (Figs 3 and 4). The first contained the 12 haplotypes of the NorthX and NorthP clades, which differed by a minimum of eight substitutions. The second contained only the 19 haplotypes of the Hawaii clade, which were separated by a minimum of 12 substitutions from the NorthX and NorthP clades. We chose not to implement the ParsPlus1 criterion (Templeton *et al.* 1992; Crandall 1996) to unite these two networks because many nonparsimonious loops would have been introduced into the analysis. Furthermore, we know that the patterns that would be created by uniting a Hawaii Island clade with a principally northern island clade are the result of dispersal over the ocean channel separating Maui from Hawaii.

NCA revealed a widespread lack of correlation between haplotypes and geography, although it did allow the inference of process in one case (Fig. 5). The exact test and NCA of Hawaii Island haplotypes revealed no geographical genetic structure (results not shown). Within the NorthX and NorthP clades, clade 2-1 (NorthP), clade 2-2 (NorthX), and the total cladogram all showed significant geographical associations of haplotypes, according to the exact test. However, significant geographical distances were only observed in clade 2-1 (NorthP). Evaluation using the inference key was as follows: 1-y, 2-y, 3-y, 5-n, 6-n, 7-n, 8-y: restricted gene flow/dispersal but with some long-distance dispersal over intermediate areas not occupied by the species. This applies specifically to the unique haplotype from the Kipahulu Valley, which likely arrived there by dispersal.

The exact test was not significant for clade 1-6, of which all but one individual are from Maui Nui (Fig. 3). This

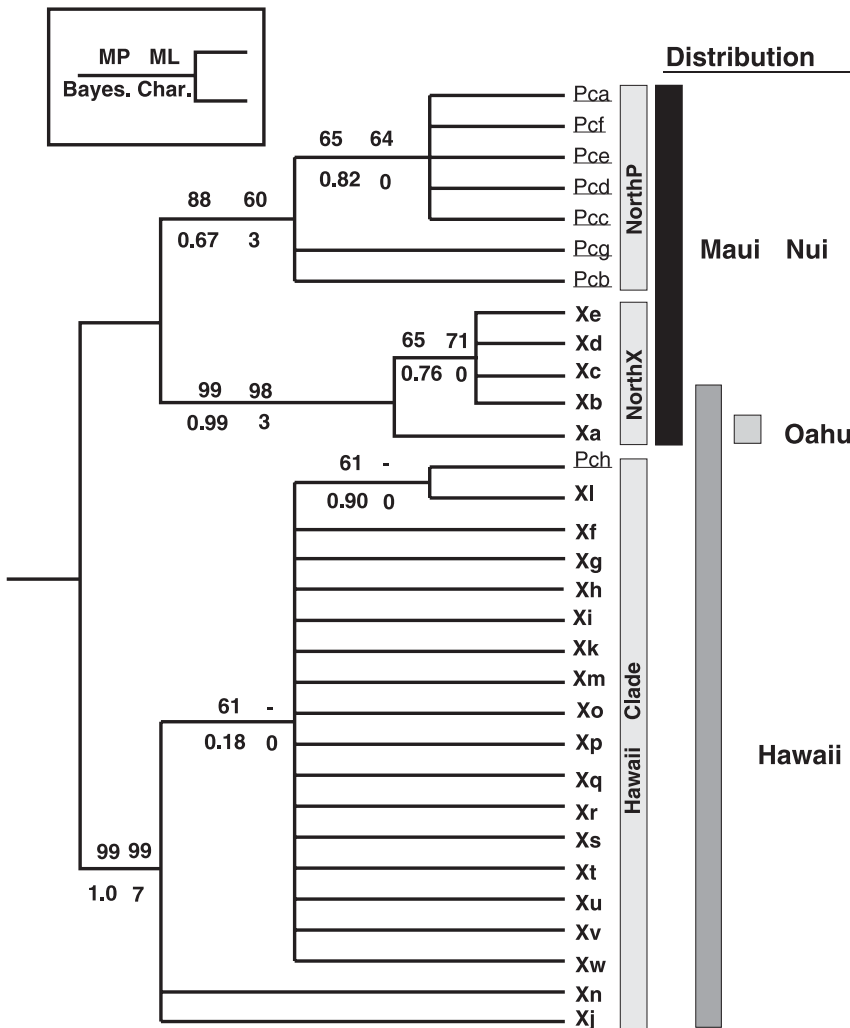


Fig. 2 Summary tree from three separate and unrooted phylogenetic analyses. Equally weighted maximum parsimony bootstrap values (1000 replicates) are shown above the lines, on the left. Appropriate model maximum-likelihood bootstrap values are shown above the lines, on the right (HKY + I; 100 replicates). Bayesian posterior probabilities are shown below the lines, on the left (HKY + I). The numbers of diagnosable characters for clades are shown below the lines, on the right. Only bootstrap percentages above 60% are shown. The root location was inferred using the method of Lundberg (1972; see text for details), and ML analysis.

indicates that there is no correlation between the geography of Maui Nui and these haplotypes. The exact test was significant for clade 2-2, which adds only one haplotype, Xa ($n = 36$), and one population, Oahu ($n = 20$), to clade 1-6. The Oahu clade is monomorphic for haplotype Xa, which is likely the result of documented recent bottlenecks (Englund 2001). In order to test whether addition of this Oahu population led to the significance of the exact test for clade 2-2, we ran the NCA again, this time excluding the Oahu population. In this case, the exact test was not significant, indicating that all haplotypes (including Xa) in *M. xanthomelas* are randomly distributed across Maui Nui.

AMOVA

The AMOVA revealed that 89% of the genetic variation was a result of differences between the three groups (Oahu and Maui Nui *M. xanthomelas*, Maui Nui *M. pacificum*, and Hawaii individuals) and only 9% of it was within these

groups. Pairwise population F_{ST} s were high between these groups, and quite low within the Hawaii group. F_{ST} s were low to moderate within the northern *M. xanthomelas* and *M. pacificum* groups (Table 2). The F_{ST} between the Maui and Molokai *M. pacificum* was 0.52. Within Maui Nui, *M. xanthomelas* F_{ST} s ranged from 0.0 to 0.31. Gene diversity, pairwise differences, nucleotide diversity and theta (S) for the Hawaii clade were significantly higher than for either of the other two clades (Table 3).

Discussion

By land or by sea?

As a substantial body of phylogeographic studies of Hawaiian terrestrial invertebrates has accumulated in recent years (Piano *et al.* 1997; Holland & Hadfield 2002; Jordan *et al.* 2003; Vandergast *et al.* 2004), it has become more and more clear that genetic differentiation below

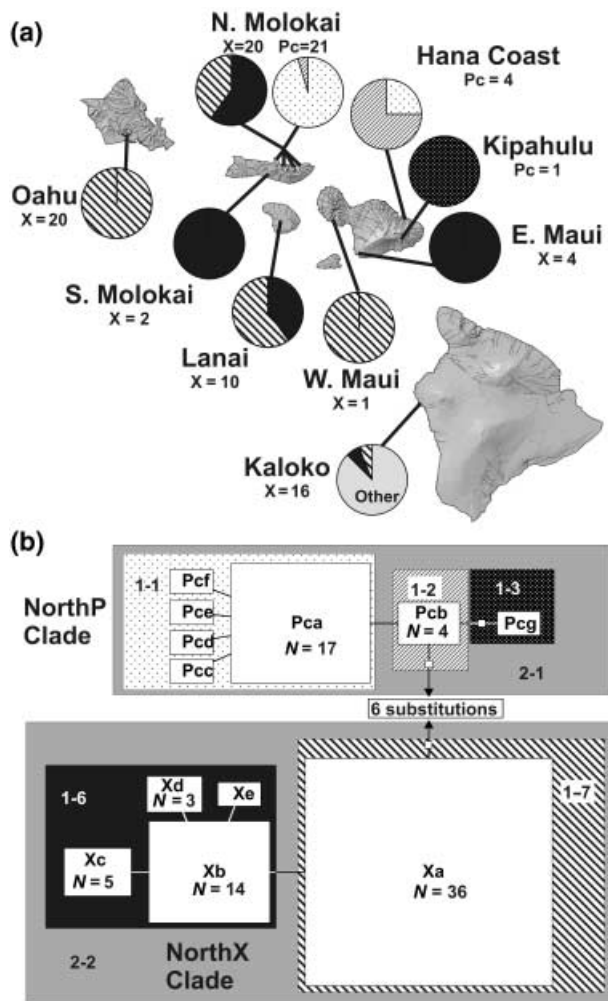


Fig. 3 Sampling sites with haplotype distributions for *Megalagrion xanthomelas* and *Megalagrion pacificum* from two strongly supported clades (see Fig. 2) that could be joined with 95% certainty using the rcs parsimony criteria of Templeton *et al.* (1992). (a) Map of main Hawaiian Islands excluding Kauai and Niihau. The population frequency of one-step nesting clades of COII haplotypes are noted by pie charts whose shading pattern corresponds with one-step clades in part b below. Sample sizes for each species are noted (X, *M. xanthomelas*; Pc, *M. pacificum*). Widespread clades with multiple haplotypes include clade 1-1, where haplotype Pcd was from the Hana Coast of Maui and all others were from N. Molokai, and clade 1-6, where haplotype Xb occurred on Molokai and Maui, haplotype Xc occurred on N. Molokai and Lanai, haplotype Xd was found only in the coastal ponds of E. Maui, and haplotype Xe was found only on N. Molokai. (b) Nesting structure imposed on rcs networks and used as the basis for nested clade analysis. White boxes represent individual haplotypes. Box sizes correspond to the number of individuals bearing each haplotype. Smallest haplotype boxes without sample sizes noted represent single individuals. Lines between boxes represent single nucleotide substitutions. Tiny unlabelled boxes represent intermediate haplotypes that were not found among the organisms sampled.

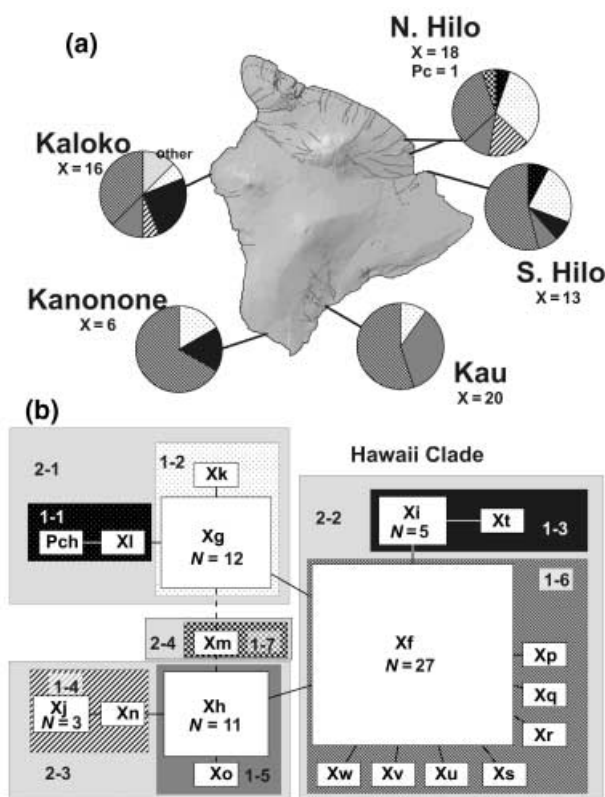


Fig. 4 Sampling sites with haplotype distributions for *Megalagrion xanthomelas* and *Megalagrion pacificum* from a strongly supported clade found only on Hawaii (see Fig. 2) that could be joined with 95% certainty using the rcs parsimony criteria of Templeton *et al.* (1992). (a) Map of the island of Hawaii. The population frequency of nesting clades of COII haplotypes are noted by pie charts whose shading pattern corresponds with one-step clades in part b below. Sample sizes for each species are noted (X, *M. xanthomelas*; Pc, *M. pacificum*). (b) Nesting structure imposed on rcs networks and used as the basis for nested clade analysis. White boxes represent individual haplotypes. Box sizes correspond to the number of individuals bearing each haplotype. Smallest boxes without sample sizes noted represent single individuals. Lines between boxes represent single nucleotide substitutions.

the species level is driven by recent and local processes (e.g. dispersal, local vicariance and barriers, and gene flow), including selection (Craddock & Carson 1989). This stands in contrast to traditional higher-level Hawaiian biogeography and Canary Island phylogeography, where stepping-stone progression from old to young islands and ecological radiations are common (Funk & Wagner 1995; Roderick & Gillespie 1998; Juan *et al.* 2000). The current study contributes to the mounting evidence for the importance of recent and local processes to intraspecific genetic variation in Hawaiian terrestrial invertebrates.

Two lines of reasoning, both resulting from comparison of patterns on Maui Nui and Hawaii, underscore the importance of Pleistocene island connections in structuring mtDNA genetic diversity in these species. The first is the

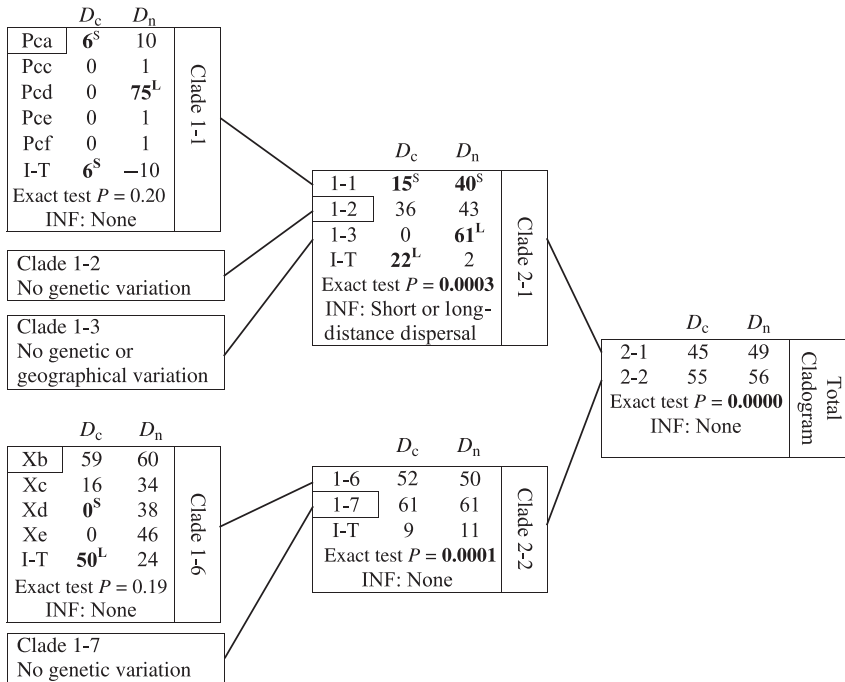


Fig. 5 Results of nested clade analysis (NCA). Nesting clades are represented as boxes. Lower level clades are found on the left, and are nested in higher level clades as indicated by lines. Haplotypes in 1-step clades correspond to Table 1. Clade distances (D_c) and nested clade distances (D_n) for each nested clade are arranged in columns. Bolding and superscript S and L indicate that the distance was significantly small or large, respectively. Results of the exact permutation test for each clade are indicated in the bottom of each box, as well as the process inferred to be responsible for the observed pattern, given the inference key of Templeton (1998). Private boxes indicate interior clades.

apparent rarity of overwater crossings, as manifested by the genetic isolation of Hawaii Island, which appears to have been colonized by *Megalagrion xanthomelas* and *Megalagrion pacificum* as few as three times and whose endemic haplotypes have likely never been established on other islands. The second concerns the results of the AMOVA and the exact tests from the NCA, which found little to no geographical structuring of haplotypes in *M. xanthomelas* within Maui Nui or Hawaii (Table 2). Hawaii island is large, and yet panmixia seems to exist between its populations, suggesting that *M. xanthomelas* disperses well across land. Since the islands of Maui Nui are now separated by water connections, we suggest that apparent panmixia there is a relict of land connections between them that existed as recently as 15–20 ka. Historical connections between islands have had a homogenizing effect and seem to be more important than current island isolation in structuring genetic diversity.

In particular, we did not find the vicariance patterns proposed by Funk & Wagner (1995), Roderick & Gillespie (1998), and Craddock (2000) (scenario 1 in the Introduction). Nor did we find complete panmixia between islands without respect to ocean channels between them (scenario 3). Our results, like those of Piano *et al.* (1997), are in accord-

ance with the intermediate scenario 2, which posits that overland dispersal is common and overwater dispersal is rare. Davies & Bermingham (2002) have also noted that sea channels between islands can represent significant barriers to gene flow, in their study of two Caribbean butterflies that disperse well over land.

Island size and genetic diversity

As sea levels have changed, the size and shape of available habitat on islands have likely changed as well. At least two species responses to these changes are possible, depending upon the shape of the island. First is the habitat ‘shift’ response. If falling sea levels do not uncover significant amounts of usable terrain, species might simply track suitable habitat as it changes location and elevation (Coope 1979) without any significant variation in range size. This would be the case, for example, if suitable habitat shifted up or down an island’s slopes. Alternately, under the ‘expansion/contraction’ response, if newly exposed habitat is suitable, species may increase their range and population sizes. However, under certain circumstances the needed habitat might diminish or disappear, leading to population

Table 3 Metrics of genetic diversity for each population group, calculated using ARLEQUIN

	Gene diversity	Mean number of pairwise differences	Nucleotide diversity	Theta (S)	SD
NorthP	0.563 ± 0.108	0.785 ± 0.589	0.00119 ± 0.00100	1.8344	0.8759
NorthX	0.571 ± 0.058	0.774 ± 0.574	0.00117 ± 0.00096	0.8609	0.4736
Hawaii	0.810 ± 0.035	1.419 ± 0.878	0.00215 ± 0.00147	3.5074	1.2146

contraction and possible extinction. Episodes of expansion and contraction might occur in the same population as conditions vary over time.

Distinguishing between habitat shift and expansion/contraction may be difficult and involve an overlay of population genetic diversity onto island topography, as well as specific results of statistical phylogeographic methods like NCA. In general, island populations occupy highly restricted distributions compared to their continental counterparts, and may have lower genetic diversity to work with. Under the shift response, we would expect no dramatic change in the effective population size (N_e) and thus no change in haplotype diversity over time. Under the expansion/contraction response, however, large portions of suitable habitat are lost to rising sea levels and population sizes may plummet, only to rise again with the next ice age. When these cycles are repeated many times, as they were during the climatic fluctuations of the Pleistocene, loss of genetic diversity may be even more severe. In such cases, N_e is best estimated using the harmonic mean of the census population sizes. The harmonic mean is dominated by the smaller values (Crow & Kimura 1970), and so N_e may be surprisingly small. Dispersal to newly exposed terrain during global cooling will establish populations with further reductions in genetic diversity through founder events and bottlenecks, although these patterns may be difficult to identify over small geographical distances, for highly dispersive taxa, or without genetic markers that evolve at a suitable rate. Therefore, in general we would expect genetic diversity on islands to be correlated with the amount of suitable habitat available in refugia during interglacial periods, when population sizes are small. If geographical distances are large enough, or dispersal is rare enough to prevent panmixia, signatures of population expansion may be identified by NCA or frequency-based methods.

Mitochondrial genetic patterns of *M. xanthomelas* and *M. pacificum* are consistent with this expansion/contraction response in several ways, although any conclusions are tentative. First, Oahu and Maui Nui populations of *M. xanthomelas* and *M. pacificum* display much lower mtDNA diversity than Hawaii Island *M. xanthomelas* populations (Table 3). This pattern could be explained by several possible scenarios including founder effects and past population bottlenecks. The most plausible explanation, however, involves an interaction between sea level fluctuation and the unique topography of these islands. As mentioned in the Introduction, the island of Hawaii is characterized by volcanic cones that rise steeply from the ocean and continue to fall off sharply below sea level. The islands of Maui Nui rise from a large, shallow platform (see maps at <http://geopubs.wr.usgs.gov/i-map/i2809/>). During low sea stands, the total area available to *M. xanthomelas* and *M. pacificum* on Hawaii did not likely change much, while

large areas of suitable habitat became available on Maui Nui (Price & Elliott-Fisk 2004). When sea levels climbed, population sizes likely decreased dramatically on Maui Nui as damselflies were forced from preferred lowland and coastal wetland habitats to streams.

Repeated sea level fluctuations throughout the Pleistocene may have caused Maui Nui damselfly populations to undergo many boom and bust cycles, resulting in a lower overall effective population size and subsequent loss of haplotype diversity. In contrast, Hawaii *M. xanthomelas* populations maintained higher genetic diversity in spite of sea level changes. This is probably because Hawaii had suitable habitat that simply moved up and down its volcanic cones in a 300–400 m elevational band beginning at sea level. Damselfly populations likely underwent a shift response, moving in concert with this shifting habitat without suffering dramatic reductions in effective population size and consequent drops in haplotype diversity. We also see the historical contingency of island genetic patterns in that different island topographies led to different responses to sea level change.

Two potential cases of introgressive hybridization also offer intriguing hints about the effects of sea level change. The first case, that of the single Hawaii Island *M. pacificum* which carries *M. xanthomelas* mtDNA and *M. pacificum* nuclear DNA, is clear-cut and likely recent. This case of introgression may be the result of the extremely low population size of *M. pacificum*, which is surrounded by large populations of *M. xanthomelas* (R.A. and D.F., unpublished). Several authors have pointed out that hybridization is more likely to occur under these circumstances, that is, on the geographical edge of a species' range, when its populations are small and surrounded by a numerically dominant species (e.g. Arnold 1997; Seehausen 2004). This case establishes that hybridization between these species is possible.

The second case stems from more ancient discordance between the nuclear and mtDNA phylogenies for these species. It involves introgression of mtDNA between Oahu and Maui Nui populations of both species, and may have been facilitated by range shifts as the lowland and coastal habitats preferred by *M. xanthomelas* were lost to rising seas, forcing them into low and midstream reaches preferred by *M. pacificum*. There are several lines of evidence to suggest that the entire mtDNA genome of *M. pacificum* was replaced via introgression by *M. xanthomelas* mtDNA. The mtDNA phylogeny of the genus *Megalagrion* shows that these two species occur at the end of an exceptionally long branch which originated 7.9 Ma and did not split until immediately prior to the *M. xanthomelas* and *M. pacificum* clades at 1.2 Ma (Jordan *et al.* 2003). This long, unbroken branch is likely due to the extinction of ancestral species and island populations, a hypothesis that is consistent with the collection records we have (Fig. 1). The age of these long branches also renders unlikely the possibility that

these species arose on a young island and dispersed back up the chain or that their shared polymorphism is ancestral. Since the *M. xanthomelas* populations on the island of Hawaii are not in sympatry with significant numbers of *M. pacificum* and so probably never experienced introgression, the *M. xanthomelas* haplotype there may be closest to the ancestral *M. xanthomelas* haplotype. Furthermore, the original *M. pacificum* haplotype would have branched much earlier than Jordan *et al.*'s (2003) 1.2 Myr estimate from the mtDNA. Taking into account all relevant evidence, this introgressive hybridization likely occurred during the Pleistocene on Oahu Nui or Maui Nui.

The replacement of an entire species' mtDNA genome with introgressed mtDNA is not unheard of in Hawaii, having been well documented in the *Laupala* crickets (Shaw 2002). Establishing the direction of introgression conclusively would require that we know the ancestral mtDNA types for *M. xanthomelas* and/or *M. pacificum*. Unfortunately, the extinction of these species on the older Hawaiian Islands (Fig. 1) makes genetic sampling difficult. Efforts to sequence mtDNA from museum specimens representing extinct populations failed (S.J., unpublished).

Pleistocene glaciation and islands

Phylogeographic patterns of Hawaiian damselflies on Maui Nui and Hawaii suggest an island analogue to continental processes that lead to northern genetic purity and southern richness. That is, we find a class of populations (Hawaii island) that are able to maintain their genetic diversity throughout glacially induced range shifts (due to rising sea levels), and a class of populations (Maui Nui) that suffer a loss of genetic diversity from a series of population reductions, also associated with global glaciation patterns.

Further work is needed to examine specific predictions that are suggested by analysis of Hawaiian damselflies. First, the idea that genetic diversity will be correlated with total area available to taxa in interglacial refugia (large island diversity, small island purity) is only suggested by the present study. A corroboration of multiple independent data sets is needed before this idea rises to anything like the level of certainty accorded to Hewitt's continental dictum (1996). Second, the specific effects of island topography on available habitat need to be examined for a variety of taxa on Maui Nui and Hawaii before we can be certain that differences in genetic diversity are related to habitat size. Similarly, the specific effects of island topography on introgressive hybridization need to be examined for more taxa before we can say whether Pleistocene fragmentation has facilitated hybridization. Finally, additional phylogeographic studies of Hawaiian terrestrial animals will allow us to understand the degree to which predictions based on higher-level biogeography apply to intraspecific variability (Roderick & Gillespie 1998). A potentially rich

addition to our knowledge of island biogeography will occur as phylogeographic data sets accumulate for Hawaiian taxa with variable ecological and behavioural attributes.

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This paper represents a portion of Steve Jordan's PhD research completed at the University of Connecticut. His research on the systematics and phylogeography of Polynesian damselflies is ongoing at Bucknell University. Chris Simon has worked extensively in Polynesia and has broad interests in systematics, molecular systematics, and their application to questions of insect evolution and biogeography. David Foote uses insects to address a variety of conservation and ecological questions for the USGS at Hawaii Volcanoes National Park. Ron Englund is an aquatic ecologist with extensive experience working in Utah, Hawaii, and French Polynesia.
