Molecular biogeography and host relations of a parasitoid fly

David A. Gray[1†], Henry D. Kunerth[2,3†], Marlene Zuk[3], William H. Cade[4], and Susan L. Balenger[5†]

[1]Department of Biology, California State University Northridge, Northridge, CA, 91330, USA

[2]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, 14853, USA

[3]Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN, 55108, USA

[4]Department of Biological Sciences, University of Lethbridge, Lethbridge, AB, T1K 3M4, Canada

[5]Department of Biology, University of Mississippi, University, MS, 38677, USA

[†]These authors contributed equally to this work.

Correspondence: David Gray, Department of Biology, California State University Northridge, 18111 Nordhoff Street, Northridge, CA 91330-8303; dave.gray@csun.edu

Abstract. Successful geographic range expansion by parasites and parasitoids may also require host range expansion. Thus the evolutionary advantages of host specialization may trade off against the ability to exploit new host species encountered in new geographic regions. Here we use molecular techniques and confirmed host records to examine biogeography, population divergence, and host flexibility of the parasitoid fly, *Ormia ochracea* (Bigot). Gravid females of this fly find their cricket hosts acoustically by eavesdropping on male cricket calling songs; these songs vary greatly among the known host species of crickets. Using both nuclear and mitochondrial genetic markers, we (1) describe the geographical distribution and sub-division of genetic variation in *O. ochracea* from across the continental United States, the Mexican states of Sonora and Oaxaca, and populations introduced to Hawaii; (2) demonstrate that the distribution of genetic variation among fly populations is consistent with a single widespread species with regional host specialization, rather than locally differentiated cryptic species, (3) identify the more-probable source populations for the flies introduced to the Hawaiian islands; (4) examine genetic variation and sub-structure within Hawaii; and (5) discuss specialization and lability in host-finding behavior in light of the diversity of cricket songs serving as host cues in different geographically separate populations.

Key Words: parasitoid, host-specialization, range expansion, *Gryllus*, *Teleogryllus*, *Ormia*

Introduction.

Evolutionary specialization is often viewed as a double-edged sword: specialization may facilitate efficient exploitation of favored resources, but may also inhibit exploitation of novel resources. Specialization has often been viewed as an evolutionary 'dead-end' (Raia and Fortelius, 2013, Jaenike, 1990, Kelley and Farrell, 1998), although recent research has revealed considerable flexibility among specialist lineages and occasional 'reversals' from specialized to more generalized niches (Vamosi et al., 2014, Gompert et al., 2015). The retention of evolutionary lability may be especially relevant for geographic range expansion; indeed 'generalist' species are often among the most invasive (Romanuk et al., 2009) – a pattern found among plants, arthropods, mammals and birds (Higgins and Richardson, 2014, González-Suárez et al., 2015, Blackburn and Duncan, 2001, Snyder and Evans, 2006). For specialist species to expand their geographic range, they must readily encounter suitable resources, exhibit phenotypic plasticity enabling adoption of novel resources, and/or show rapid evolutionary adaptation.

Parasitoid insects, especially Ichneumonid and Braconid wasps (Hymenoptera) and Tachinid flies (Diptera), are especially illuminating for studies of host specialization, ranging from extreme generalists to extreme specialists (Quicke, 2014, Stireman et al., 2006). Some species are sufficiently host specific to be used for classical biological control of pests (Parkman et al., 1996, Vargas et al., 2007), others routinely utilize a broad range of hosts (Stireman, 2005, Tschorsnig, 2017, Arnaud, 1978), and in other

cases, presumed generalists are later revealed to be complexes of cryptic specialists (Smith et al., 2008).

Within the ca. 9000 species of Tachinids, the Ormiini tribe represents a small group (ca. 68 described species) of highly specialized flies (Sabrosky, 1953a, Sabrosky, 1953b, Lehmann, 2003). Several specializations are noteworthy for the entire group (so far as is known): all are parasitoids of crickets or katydids (Ensifera, Orthoptera); all locate their (principally male) hosts using a specialized ear (Edgecomb et al., 1995, Hedwig and Robert, 2014) to eavesdrop on their male host's mating song (Lehmann, 2003, Cade, 1975, Allen, 1995); all have sclerotized planidiform larvae which are somewhat mobile and actively burrow into the host (Cantrell, 1988, Adamo et al., 1995b). Within this group, all genera with known hosts parasitize katydids (Tettigoniidae); in the genus *Ormia* most species parasitize katydids but three species attack crickets and mole crickets (Gryllidae and Gryllotalpidae) (Lehmann, 2003). The shift from katydids to crickets and mole crickets represents a significant shift in female fly hearing towards lower frequency sounds (ca. 4-5 kHz in crickets and ca. 2-3 kHz in mole crickets) than are typical of most katydids (often >>10kHz). Utilization of katydids with relatively low frequency calls may have facilitated the evolutionary transition to crickets and mole crickets. For example, certain katydid hosts of Ormiines have relatively low frequency calls, e.g. ca. 5-6 kHz in *Sciarasaga quadrata* (host of *Homotrixa alleni*) (Allen et al., 1999); ca. 7 kHz in *Neoconocephalus robustus* (host of *O. brevicornis*) (Nutting, 1953); ca. 8 kHz in *Orchelimum pulchellum* (one of several hosts of *O. lineifrons*) (Shapiro, 1995).

Within *Ormia*, *O. ochracea* has been most extensively studied. Peak sensitivity of female hearing closely matches or is at slightly higher frequencies than typical male calling song (Robert et al., 1992). The current geographic range attributed to this species extends from Florida (Walker and Wineriter, 1991), across the southern Gulf States (Henne and Johnson, 2001), into Texas (Cade, 1975), Arizona (Sakaguchi and Gray, 2011), California (Wagner, 1996), and Mexico (Sabrosky, 1953b); throughout this range it parasitizes various species of *Gryllus* field crickets (see below). In addition, *O. ochracea* was introduced to Hawaii by at least 1989 (Evenhuis, 2003), where it parasitizes *Teleogryllus oceanicus*, itself introduced to Hawaii by at least 1877 (Kevan, 1990) and possibly earlier, perhaps facilitated by Polynesian settlement (Tinghitella et al., 2011). Localized populations of *O. ochracea* show varying degrees of host specialization: flies in Florida almost exclusively parasitize *Gryllus rubens* (Walker, 1993, Walker and Wineriter, 1991); flies in Texas primarily parasitize *G. texensis* (Cade, 1975); flies in Arizona regularly parasitize multiple *Gryllus* species (Sakaguchi and Gray, 2011); flies in southern California primarily parasitize *G. lineaticeps* (Wagner, 1996, Wagner and Basolo, 2007); as noted above, Hawaiian flies parasitize *T. oceanicus*. Remarkably, playback experiments in Florida, Texas, California, and Hawaii, which simultaneously presented the songs of *G. rubens*, *G. texensis*, *G. lineaticeps*, and *T. oceanicus*, revealed that each fly population showed a significant (but not exclusive) preference for the song of its primary local host species of cricket (Gray et al., 2007). This suggests an even further degree of host specialization in these flies – possibly indicative of cryptic host races or species as has been found in other Tachinids (Smith et

al., 2008, Smith et al., 2006). Determining the extent to which geographic and host range subdivision is coupled with genetic subdivision is thus one of the goals of this study.

Successful establishment of *O. ochracea* in Hawaii represents a significant expansion of both the geographic and host range of the fly. How can such a specialist invade, switch to a novel host with a strongly divergent song structure, and in the course of a few decades come to prefer that novel host's song to the songs of ancestral hosts? Two of our aims in this paper are to use mitochondrial and nuclear markers both to examine genetic variation within Hawaii and to identify the more-likely continental source population(s) of those Hawaiian flies, and thereby the most likely types of recent ancestral host songs. This necessitates broad sampling of continental populations, and we therefore expand upon the previous work in the USA and include flies from populations in both northern and southern Mexico, as well as catalog the confirmed host species and their songs in each of these areas. We apply standard phylogeographic analyses to mitochondrial DNA sequence data, including outgroup species of *Ormia*, and we adopt a population genetic approach to analysis of microsatellite nuclear markers.

Methods.

Fly collection

We collected flies at mesh screen and/or bottle traps using playbacks of cricket songs (Walker, 1989); we also collected a small number of flies at lights or as they emerged from field-collected crickets. Table 1 provides details of locations and dates of sampling. Collected flies were preserved in ethanol until DNA extraction and further analysis. We extracted DNA using a Qiagen DNeasy tissue kit according to the manufacturer's

instructions. We used entire flies as source tissue for all of the mainland and 13 of the Hawaiian flies, and head and thorax tissue for the remainder of the Hawaiian flies. In theory, the whole tissue extractions could include DNA from larvae, although the amounts of such DNA would be trivial compared to maternal DNA. We quantified DNA using a Nanodrop system and adjusted concentrations to between 20 and 75 ng/ul.

Genetic Markers & Analysis

We analyzed population structure using both mitochondrial and nuclear markers. For mtDNA, we analyzed a section of *Cytochrome C Oxidase subunit I* (hereafter COI) PCR amplified in two overlapping fragments with 'universal' primer pairs Jerry-Pat and Ron-Nancy (Simon et al., 1994), resulting in 1111 bp after alignment. In addition, we developed nuclear microsatellite markers *de novo* for this project. Marker discovery was performed by 454 sequencing at the Cornell University Life Sciences Core Laboratories Center with further validation done by SLB and HDK. We identified and tested 17 msat markers from this dataset consisting of 3, 4, and 6 bp repeats. PCR conditions followed a 'touchdown' protocol of 95° for 40 seconds, 66° for 45 seconds, and 72° for 45 seconds. The annealing step was reduced by one degree every cycle for the first seven cycles. Cycles 8-35 followed a pattern of 95° for 40s, 58° for 45s, and 72° for 45s. PCR products were stored at -20°C until genotyped. Individuals were genotyped at microsatellite loci by the University of Minnesota Genomics Center on an Applied Biosystems 3730xl DNA Analyzer. We scored alleles for fragment size manually using Peak Scanner 2.0 software. Multiple independent analysts scored the same products to

assure veracity of the calls. If no clear designation could be made or alleles did not amplify, we scored the data as missing.

Bioinformatic Analyses

Prior to analysis of microsatellite fragments, we filtered individuals and loci for missing data. A strict cutoff of >25% missing data led to the exclusion of 6 loci. Following this filter, we excluded any individuals with missing data at 3 or more loci. The final dataset included 274 individuals genotyped at 11 loci with between 6 and 17 alleles per locus (Table 2). To estimate the number of alleles and private alleles accurately given unequal sample sizes per population, we performed a rarefaction analysis using HP-Rare (Kalinowski, 2005). We visualized population genetic variation using a discriminant function analysis of principal components (DAPC) with 80 principal components and 4 discriminant functions using the adegenet (Jombart, 2008, Jombart and Ahmed, 2011) and pegas (Paradis, 2010) packages in R.

To visualize genetic structure, we implemented the Bayesian analysis program STRUCTURE v2.3.4 using an admixture model with correlated allele frequencies. We used a burn-in of 50,000 steps and 100,000 MCMC iterations. We conducted separate runs for the full dataset, a dataset with the Hawaiian samples excluded, and a dataset of only Hawaiian samples. For the full dataset, we performed 5 runs each for k = 2-9. To infer the likely number of genetic clusters, we used both the Ln estimated probability of the data from STRUCTURE and the Evanno method utilizing Δk (Evanno et al., 2005).

We calculated pairwise estimates of Fst (Weir and Cockerham, 1984) and Nei's genetic distance between populations using the R packages adegenet and ade4 (Chessel et al., 2004), and we calculated expected and observed heterozygosity using adegenet.

We built a mitochondrial haplotype network using 55 haplotypes from 1111 bp of COI sequences from 275 individuals using the R package pegas (Paradis, 2010) with default parameters.

Host Ranges & Songs

To provide context for understanding the degree of host specialization, we present in this paper the songs of confirmed hosts in each of the geographic regions studied. We present only hosts confirmed to be naturally parasitized by development of *O. ochracea* from field-collected crickets. We suspect that a few additional host species will be confirmed in the USA, especially if the species is only occasionally parasitized, and we expect that many more species are parasitized in southern and central Mexico; this reflects the status of current knowledge of *Gryllus* systematics and the extent of field sampling. Many of the confirmed host species are not yet officially described (DB Weissman and DA Gray, in prep.); to provide continuity within the literature we use provisional manuscript names here and note that the names are disclaimed as unavailable per Article 8.3 of the ICZN.

In an attempt to quantify relative song differences, we created a Euclidean song distance matrix using *matrix <- dist(songdata)* function in R. Song variables were: dominant frequency (kHz), pulse rate, pulses per chirp or trill (ln transformed), pulse duty cycle, song type (chirp, trill, stutter-trill, complex stutter-trill), chirps per trill (for stutter-

trillers), as well as introductory pulses per trill and introductory pulse rate (for complex stutter-trillers). Prior to matrix calculation, the raw song data were normalized as z-scores. The resulting song distance matrix has the advantage of objectively showing unit-less quantitative differences among species, but has the disadvantage that the different song features are not weighted by their perceptual importance to *O. ochracea*, which would be preferable but is not currently possible.

Results.

Nuclear and mitochondrial genetics

Following filtration at missing data cutoffs, 274 individuals and 11 loci were included in the final msat dataset, with 1.86% data missing. Heterozygosity across all individuals was 50.9%. The Hawaiian populations showed a drastic decrease in heterozygosity (Table 3). The rarefaction analysis also suggested a substantial decrease in both total and private allelic diversity within the Hawaiian populations (Table 2).

Analysis of Nei's genetic distances documented a clear split between Hawaiian and mainland populations (Table 4), with Hawaiian populations more similar to western mainland populations. Longitude explained the primary axis of variation among the mainland populations, with a clear east-west gradient evident in both the DAPC and mtDNA haplotype network (Fig. 1), as well as in the pairwise Fst and Nei's distances (Table 4).

For the full dataset, STRUCTURE analyses indicated the strongest support for k=2 genetic clusters (Fig. 2) separating Hawaiian from mainland populations, however support for k=3 clusters was also high, which further divided the mainland populations

into eastern and western subsets (Fig. 2). STRUCTURE plots for within Hawaii (k=2 and k=3) and mainland (k=2, k=3, and k=6) are in Supplemental Materials Figs. S1 and S2.

The mtDNA haplotype network (Fig. 1b) also showed (1) low genetic variation within Hawaii, (2) affinity of the Hawaiian sequences for the western mainland (i.e. California) sequences, and (3) a longitudinal geographic structure within the mainland populations. Oaxaca had a high diversity of haplotypes shared with all other mainland populations.

Given the apparent distinctness of the Hawaiian populations, it is important to emphasize that these patterns reflect founder effects, and concomitant change in allele frequency in Hawaii, not the development of novel genetic variation in Hawaii. This is most easily seen in allele frequency histograms which show that the Hawaiian genetic variation is effectively a simple subset of the genetic variation found in western mainland populations, themselves a simple subset of the genetic variation found in Florida, Texas, and Mexico populations (see Fig. 3 for a representative locus; figures for all other loci show similar patterns and are presented as Supplemental Materials Figures S3-S12).

Host range and song structures

Confirmed host species, geographic range information, as well as host calling song type, frequency, pulse rate, and pulses/chirp are presented in Table 5. Songs of confirmed host species vary dramatically, from simple chirps to complex trills; see waveform oscillograms and frequency spectrograms in Figures 4 and 5, respectively.

The song distance matrix shows nearly 30-fold variation among species in pairwise inter-host song distance comparisons (Fig. 6). Notably, the average distance of *T. oceanicus* song from each of the other songs was about double the average distances for the continental *Gryllus* species (7.75 versus 3.85, $Z = 7.4$, $p < 0.0001$).

Discussion.

Our results suggest the following: (1) *O. ochracea* is a single widespread species with regional host specialization, not a complex of cryptic species, (2) *O. ochracea* has spread geographically into northern Mexico (Sonora) and the western USA (Arizona and California) from source populations in southern Mexico (Oaxaca) and/or the southern USA Gulf region (Florida, Texas), (3) Hawaiian flies were introduced from a western continental USA population, most likely California, potentially consisting of as few as one gravid female fly, and (4) novel song types with highly divergent song structures do not inhibit novel host exploitation. We elaborate on these results below, and discuss mechanisms of regional host song specialization.

Studies of other Tachinid groups have sometimes revealed that what was considered a single generalist species actually consists of a complex of cryptic specialist species (Smith et al., 2007, Smith et al., 2006). The regional host specialization in *O. ochracea* documented previously (Gray et al., 2007) could have been consistent with either a widespread generalist with regional host preferences or with multiple cryptic host specialists. Both the mtDNA and msat variation suggest a single species. The mtDNA sequences, although showing clear east-west geographic structure, are relatively uniform and strongly divergent from *O. depleta* and *O. lineifrons* sequences (Supplemental

Materials Figure S13). The msat data clearly show that populations strongly differentiated in host song preferences can nonetheless be genetically panmictic. Perhaps the best example of this involves flies from Florida and Texas: Gray et al. (2007) showed that Florida flies preferred *G. rubens* song over *G. texensis* song nearly 2:1 and that Texas flies preferred *G. texensis* song over *G. rubens* song 6:1. Nonetheless the pairwise Fst of 0.008 for these populations (Table 3) and the DAPC (Fig. 1a) show that these two populations are genetically rather homogenous.

Both the mtDNA and msat data also inform the broader geographic history of the fly within North America. There is a clear east-west differentiation among samples, potentially consistent with isolation by distance. Moreover, the pattern of allelic variation in the msat loci (e.g. Fig. 3) suggests serial founder effects as flies colonized the western continental USA and then Hawaii. The mtDNA similarly suggests that the older fly lineages are to be found within the southeastern USA populations (Fig. 1b; Fig. S13). In this light, it is interesting to note that Florida is home to two *Gryllus* species, *G. ovisopis* and *G. cayensis*, which lack a normal calling song (Gray et al., 2018, Walker, 1974, Walker, 2001), possibly a consequence of a prolonged history of *Ormia* parasitism in that region. In contrast, there are no non-calling *Gryllus* in western North America.

The introduction of *O. ochracea* to Hawaii appears virtually certain to have been from a western North American population. The dominant mtDNA haplotype in Hawaii is also found in California and Arizona (Fig. 1b); the msat allelic variation in Hawaii is likewise a subset of the most common alleles in California and Arizona (Fig. 3). A single introduction seems likely; the levels of genetic variation in Hawaii do not preclude the possibility that the introduction could have consisted of as few as one gravid female,

292 although it seems more plausible that multiple individuals were introduced, perhaps as
293 pupae in soil. In other systems, experimental introductions have indicated that in some
294 circumstances introductions of a single gravid female can nonetheless establish a
295 persistent population (Grevstad, 1999, Fauvergue et al., 2007). Within Hawaii, our data
296 are consistent with the spread of an introduced population among islands, rather than
297 separate introductions on each island (Supplemental Fig. S1).

298 Once in Hawaii, the adoption of *T. oceanicus* as a host represents a major shift
299 within *O. ochracea*'s repertoire of host song recognition. Quantitatively and
300 qualitatively, *T. oceanicus* song is strikingly divergent from the songs of continental
301 North American hosts (Figs. 4-6). Across the diversity of host songs, one could argue
302 that the single essential song recognition feature is a dominant frequency in the 3-6 kHz
303 range. This may be true in a strict sense, but frequency is clearly not the only song
304 recognition feature. Multiple studies have shown that the temporal pattern of sound
305 pulses is also important (Gray and Cade, 1999, Sakaguchi and Gray, 2011, Wagner,
306 1996, Wagner and Basolo, 2007, Walker, 1993). Moreover, fly populations prefer the
307 temporal structure of their most common host species, even when dominant frequencies
308 are similar (Gray et al., 2007). Perhaps most remarkably, Hawaiian *O. ochracea* preferred
309 *T. oceanicus* song over the songs of ancestral host species by a large margin (12 of 13
310 Hawaiian flies chose *T. oceanicus* song over the songs of *G. rubens*, *G. texensis*, and *G.*
311 *lineaticeps*).

312 Adoption of *T. oceanicus* as a host in Hawaii also required compatible host
313 physiology for larval development. Although mostly confined to parasitism of adult
314 males, *O. ochracea* can develop within a wide variety of crickets, including juveniles

(Vincent and Bertram, 2009) and species not normally used as hosts (Thomson et al., 2012, Adamo et al., 1995a) including *Acheta domesticus* (Paur and Gray, 2011b, Paur and Gray, 2011a, Wineriter and Walker, 1990) which is more distantly related to *Gryllus* than is *Teleogryllus* (Gray, D.A, Weissman, D.B., Lemmon, E.M., Lemmon, A.R, unpublished data). This latitude probably results from the generalized nature of the cricket immune encapsulation response (Vinson, 1990), which is exploited by Ormiines to develop a respiratory spiracle. Given this latitude, we expect that physiological compatibility with *T. oceanicus* was unlikely to be a significant factor in terms of host suitability.

Our results suggest that host specialization in *O. ochracea* is not at odds with rapid exploitation of novel hosts, as might be expected from evolutionary theory (Raia and Fortelius, 2013, Jaenike, 1990, Kelley and Farrell, 1998). But how can highly regional host song specificity (Gray et al., 2007), even to the point of flies having song preferences for certain intra-specific song variants (Gray and Cade, 1999, Sakaguchi and Gray, 2011, Wagner, 1996, Wagner and Basolo, 2007), be compatible with flexible and rapid adoption of novel hosts? If population differentiation does not explain regional host specialization, as suggested by the results presented here, then behavioral plasticity coupled with local host learning (Paur and Gray, 2011a) may be the mechanism that enables flies to escape the ‘dead-end’ of specialization.

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Author Contributions: DAG, SLB, MZ, and WHC conceived of the study and collected flies; DAG performed the mtDNA sequencing; SLB and HDK performed the msat amplification and analysis; all authors contributed to the writing and editing of the manuscript.

Data Accessibility

The COI sequence data have been deposited in GenBank with accession numbers MK522523-MK522797. Upon acceptance the msat data will be archived in Dryad.

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573

574 Table 1. Sample collection data; not all specimens were used in all analyses.

Region	**Locality**	**Dates**	**N**	**Collector(s)**
Florida	Gainesville, FL	Aug. 2002	41	DAG
Texas	San Antonio, TX	Sept. 2002	5	WHC
	Austin, TX	Sept. 2002, 2004	29	WHC & S. Walker 2002; DAG 2004
	Huntsville, TX	Sept. 2002	1	S. Walker
Arizona	Sedona, AZ	Aug. 2004	12	DAG
	Oak Creek, AZ	Aug. 2004	6	DAG
	Holbrook, AZ	Aug. 2002	1	DAG
	Verde River, AZ	Aug. 2004	3	DAG
	Madera Canyon, AZ	Aug. 2004	10	DAG
	KOFA, AZ	Sept. 2005	2	DAG
	Yuma, AZ	Nov. 2003	2	A. Izzo
	Parker Canyon, AZ	Aug. 2004	2	DAG
	Petroglyph, AZ	Sept. 2006	16	DAG
	Pinery Canyon, AZ	Sept. 2004	5	DAG
	Portal, AZ	Aug. 2003	1	DAG
Sonora	Alamos, Sonora, MX	July 2006	17	DAG
Oaxaca	San Pablo Etla, Oaxaca, MX	Nov. 2014	13	DAG
California	Malibu Creek, CA	Sept. & Oct. 2003, 2004	22	DAG
	Stunt Ranch, CA	Sept. 2002	10	DAG
	Santa Margarita Reserve, CA	Sept. 2003	5	DAG
Hawaii	Kauai, HI	Feb. & Aug. 2014	24	MZ & SLB
	Hilo, HI	Mar. 2003; Feb. & Aug. 2014	33	WHC 2003; MZ & SLB 2014
	Oahu, HI	Feb. 2014	4	MZ & SLB
Outgroups				
Ormia depleta	Gainesville, FL	Dec. 2003	2	H. Frank, via T. J. Walker
Ormia lineifrons	Gainesville, FL	Dec. 2003	2	H. Frank, via T. J. Walker

575

Table 2. Locus primer and allelic richness statistics

		Repeat locus					Mean number of alleles									Mean number of private alleles								
Locus	Primer sequence 5'-3'	motif	no.	size (bp)	Pool	Dye	HI(K)	HI(O)	HI(H)	CA	AZ	SON	OAX	TX	FL	HI(K)	HI(O)	HI(H)	CA	AZ	SON	OAX	TX	FL
Oo002	F: GTGTGTGAGCGTCTGATCTTCC	CAGC	11	191	A	VIC	2.65	3.57	3.17	3.64	4.76	4.22	4.02	4.49	5.58	0.00	0.01	0.02	0.14	0.68	0.43	1.16	0.25	0.66
	R: ATCAGCCACATTTACACTTTCCC																							
Oo007	F: TTCCTTTACTATCGTATTGGCGC	TTG	8	286	A	6-FAM	1.99	2.41	2.20	5.27	5.46	5.11	6.73	4.69	4.68	0.00	0.13	0.20	1.30	0.61	0.93	1.68	0.50	0.56
	R: AGGAAGGAAGACAAACAAACAGC																							
Oo011	F: CTGCCCTTTCACTCTTACTTGAC	AACGAC	14	395	A	PET	3.89	3.33	3.39	4.77	5.33	5.68	4.05	7.29	7.02	0.00	0.00	0.00	0.07	0.68	0.82	0.00	1.20	1.40
	R: GAGCTCCCTTGGCAAGTTAAATG																							
Oo017	F: TCAAATATGGGCTGGTTTGGATG	TGGA	10	164	A	6-FAM	2.00	2.00	1.99	3.36	4.97	5.49	6.44	5.05	6.01	0.00	0.00	0.00	0.00	0.02	0.51	1.18	0.37	1.05
	R: TGTCATGATGCAGCATAAACAAC																							
Oo022	F: AAAGGTGTTAGAAGATGTTGGCG	GGAT	9	348	B	6-FAM	3.61	2.56	2.58	6.29	7.97	6.51	8.40	7.73	7.34	0.00	0.00	0.00	0.82	1.18	0.84	1.42	1.46	1.54
	R: GATAATAGCGCTCGTAGTTGCAG																							
Oo024	F: TATGACGTGCAGCAATATGAGTG	TTG	15	164	B	PET	2.54	2.24	2.22	2.89	3.77	3.93	3.52	3.48	3.69	0.55	0.21	0.22	0.00	0.17	0.01	0.00	0.30	0.00
	R: GTGACGTACGTTTGAAATGCTC																							
Oo028	F: TCTTGTGGGTAATGGCAATTGTG	TAG	12	333	B	NED	2.00	2.41	2.18	4.69	5.97	7.04	6.68	5.30	5.76	0.00	0.41	0.20	0.64	0.58	0.16	0.39	0.18	0.21
	R: ATTTAATACGCAGCAATCCCAGG																							
Oo031	F: ACATATGGTGAGTAGTGGATCCC	AAC	11	387	B	VIC	2.70	2.43	2.31	4.14	5.16	5.25	6.54	5.77	6.91	0.00	0.00	0.00	0.00	0.48	0.41	0.28	0.22	1.21
	R: ACCAGAAGCTGTCATATAGGGAG																							
Oo032	F: TGAAGTGTGACAGTTTCTTGACG	TTG	12	416	A	VIC	2.94	3.21	3.36	5.79	5.86	4.47	6.28	7.09	6.34	0.00	0.24	0.38	1.23	0.92	0.14	1.23	1.26	0.86
	R: ACTGTCAAGGATGTTAAACTGGC																							
Oo034	F: TTCGACCAAACCCATTATGTGAC	ACA	12	182	A	NED	1.92	1.83	1.90	1.90	2.78	3.02	3.59	3.34	3.25	0.00	0.41	0.03	0.03	0.84	0.68	1.63	0.70	0.90
	R: TCCGGACTATCGAGATTGTACTG																							
Oo035	F: ATTTGCGGTGTTACTTCATTTGC	GTT	10	190	A	PET	1.33	2.06	1.43	2.64	4.72	6.14	6.08	6.28	6.98	0.00	0.41	0.00	0.07	0.63	0.79	0.34	1.15	1.87
	R: TTGCTTACCACTGTTCGCTAATC																							
						Mean	2.51	2.55	2.43	4.12	5.16	5.17	5.67	5.50	5.78	0.05	0.17	0.10	0.39	0.62	0.52	0.85	0.69	0.93
						s.d.	0.73	0.55	0.60	1.32	1.25	1.13	1.53	1.42	1.32	0.16	0.17	0.13	0.49	0.31	0.30	0.62	0.46	0.54

Table 3. Population sample sizes and heterozygosity for nuclear msat loci.

Population	Sample size	N. alleles	Heterozygosity (expected)	Heterozygosity (observed)
Kauai	20	29	0.437	0.367
Oahu	28	31	0.438	0.367
Hilo	32	34	0.401	0.321
California	32	62	0.588	0.478
Arizona	57	95	0.667	0.612
Sonora	17	70	0.677	0.588
Oaxaca	13	70	0.724	0.607
Texas	35	91	0.714	0.604
Florida	40	95	0.741	0.638

Table 4. Pairwise F_{ST} (above diagonal) and Nei's genetic distance (below diagonal) by population.

	Kauai	**Oahu**	**Hilo**	**California**	**Arizona**	**Sonora**	**Oaxaca**	**Texas**	**Florida**
Kauai	-	0.027	0.057	0.092	0.071	0.105	0.109	0.091	0.087
Oahu	0.044	-	0.047	0.088	0.079	0.099	0.095	0.100	0.098
Hilo	0.096	0.073	-	0.114	0.097	0.124	0.118	0.127	0.122
California	0.263	0.229	0.279	-	0.024	0.034	0.049	0.055	0.060
Arizona	0.282	0.267	0.291	0.088	-	0.011	0.019	0.031	0.035
Sonora	0.290	0.286	0.344	0.127	0.067	-	0.032	0.026	0.026
Oaxaca	0.327	0.305	0.365	0.235	0.151	0.169	-	0.022	0.021
Texas	0.331	0.332	0.394	0.231	0.165	0.149	0.158	-	0.008
Florida	0.337	0.336	0.388	0.273	0.187	0.167	0.171	0.045	-

650 Table 5. Confirmed hosts of *Ormia ochracea*.
651
652

Host Species	**Confirmed as Host in**	**Song type**	**Dominant Frequency (kHz)**	**Pulse rate (p/s) †**	**Pulses per chirp or trill ***	**References for host status and song data**
G. rubens	Florida	trill	4.7	50-55	100-200	(Walker and Wineriter, 1991, Vélez and Brockmann, 2006, Izzo and Gray, 2004, Blankers et al., 2015)
G. firmus	Florida, Texas	chirp	4.2	16	3-5	(Walker and Wineriter, 1991, Doherty and Storz, 1992); D. Weissman pers. com.
G. texensis	Texas, Oklahoma, Coahuila	trill	5.2	75-80	25-65	(Cade, 1975, Cade, 1981, Cade et al., 1996, Gray and Cade, 1999, Izzo and Gray, 2004, Blankers et al., 2015); DAG; D. Weissman pers. com.
G. assimilis	Texas, Oaxaca, Nuevo Leon	chirp	3.7	85	6-9	DAG; D. Weissman pers. com. (Weissman et al., 2009)
G. personatus	Arizona, Coahuila	chirp	4.0	57	6-8	DAG; D. Weissman pers. com. (Gray et al., 2016b)
G. vocalis a.k.a. Regular stutter-triller	Arizona	Fast chirp	4.8	33	3-4	D. Weissman pers. com. (Weissman et al., 1980, Sakaguchi and Gray, 2011)

G. "staccato" a.k.a. G#15	Arizona, Sonora	chirp	5.2	73	6-8	(Sakaguchi and Gray, 2011, Gray et al., 2016b); DAG
G. armatus	Arizona	stutter-trill	3.6	58	2, 15-20	(Hedrick and Kortet, 2006); DAG
G. "montis"	Arizona	chirp	3.8	22	4-5	DAG
G. "longicercus" a.k.a. G#13	Arizona	chirp	4.5	10	4-6	DAG; D. Weissman pers. com. (Gray et al., 2016a)
G. "lightfooti"	Arizona	chirp	4.5	20	4-6	DAG; D. Weissman pers. com.
G. multipulsator	Arizona, Sonora, Jalisco, Zacatecas, Sinaloa, Baja California Sur	chirp	4.1	70	12-16	A. Izzo; DAG; D. Weissman pers. com. (Weissman et al., 2009)
G. "regularis" a.k.a. G#14, Arizona triller	Arizona	trill	4.5	38	20-80	(Sakaguchi and Gray, 2011, Blankers et al., 2015); DAG
G. cohni a.k.a. G#20, Arizona stutter-triller	Arizona, Sonora	stutter-trill	4.8	25	2-8, 1-6	(Sakaguchi and Gray, 2011); DAG
G. "saxatilis" a.k.a. G#2	California, Baja California Norte	chirp	4.1	20	3-4	DAG; D. Weissman pers. com.
G. lineaticeps	California	chirp	5.1	55	6-8	(Wagner, 1996, Wagner and Basolo, 2007, Gray et al., 2016b); DAG
G. integer	California	stutter-trill	4.5	60	2-3, 15-80	(Hedrick and Kortet, 2006, Paur and Gray, 2011a, Hedrick and Weber, 1998, Weissman

						et al., 1980)
Teleogryllus oceanicus	Hawaii	complex 2-part trill // stutter-trill **	4.6	14 // 24	6-8 // 2, 8-10	(Zuk et al., 1995, Zuk et al., 1993)

† Pulse rates approximate the average at 25 ^{o}C.

* For stutter-trillers, numbers are given as pulses per chirp, chirps per trill.

** For the *T. oceanicus* 2-part song, numbers are given as trill part 1 // stutter-trill part 2.

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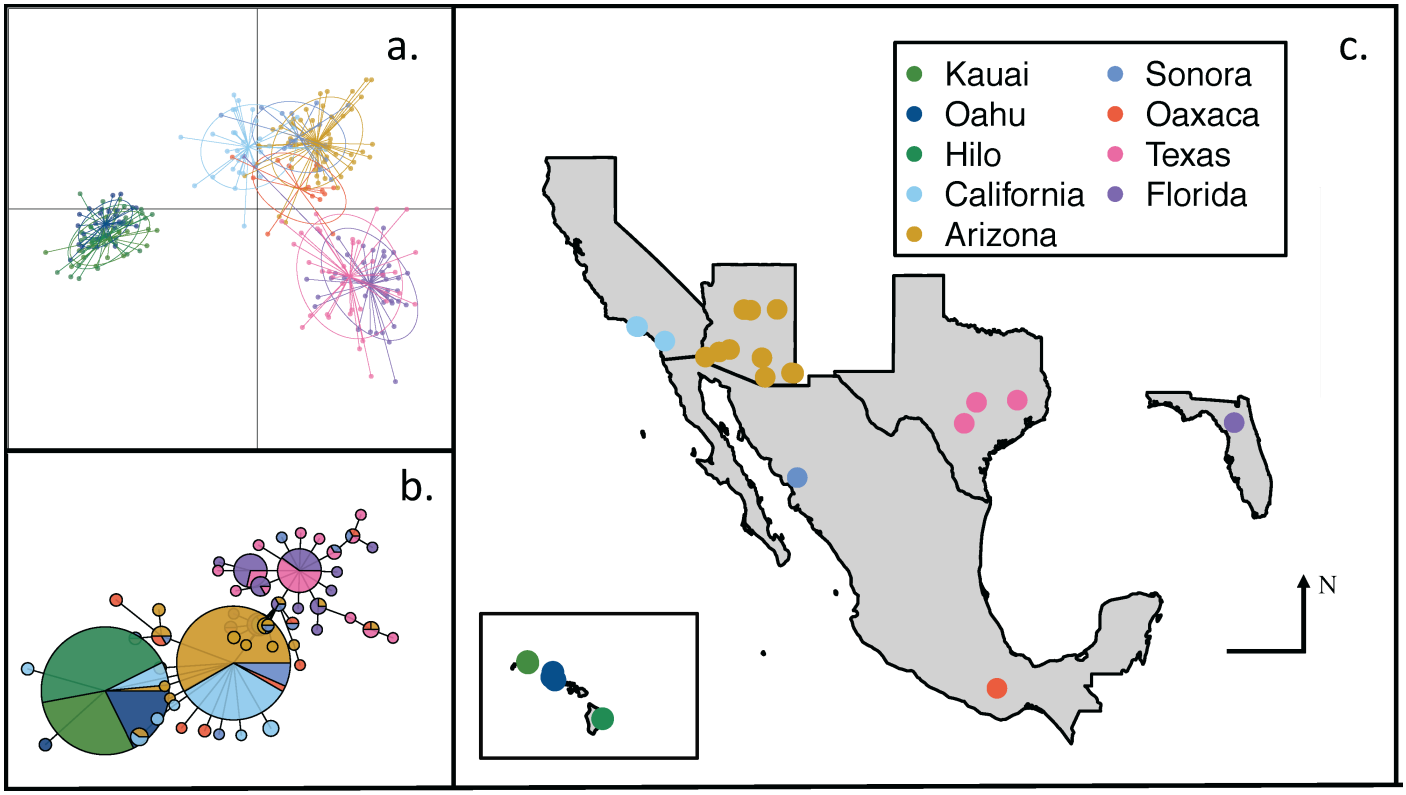


Figure 1. a) DAPC clustering analysis. Individuals are marked as points with ellipses representing 75% of the observed data. b) Haplotype network of 55 haplotypes of 1111bp of mitochondrial COI gene sequences. c) Map of collection sites.

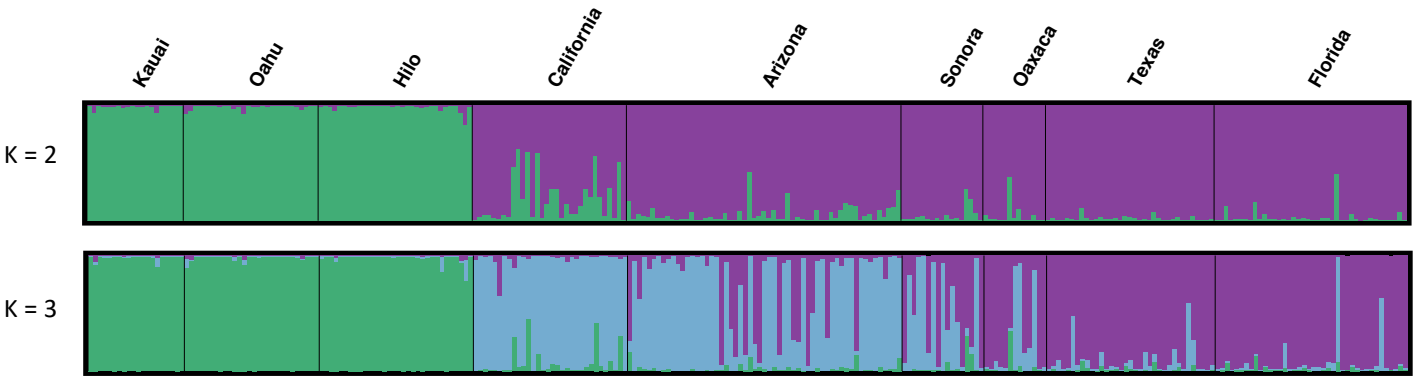


Figure 2. Bayesian clustering analysis implemented by STRUCTURE software (Pritchard et al. 2000). Top panel shows clustering into two genetic groups (K = 2) and the bottom panel shows clustering into three genetic groups (K = 3).

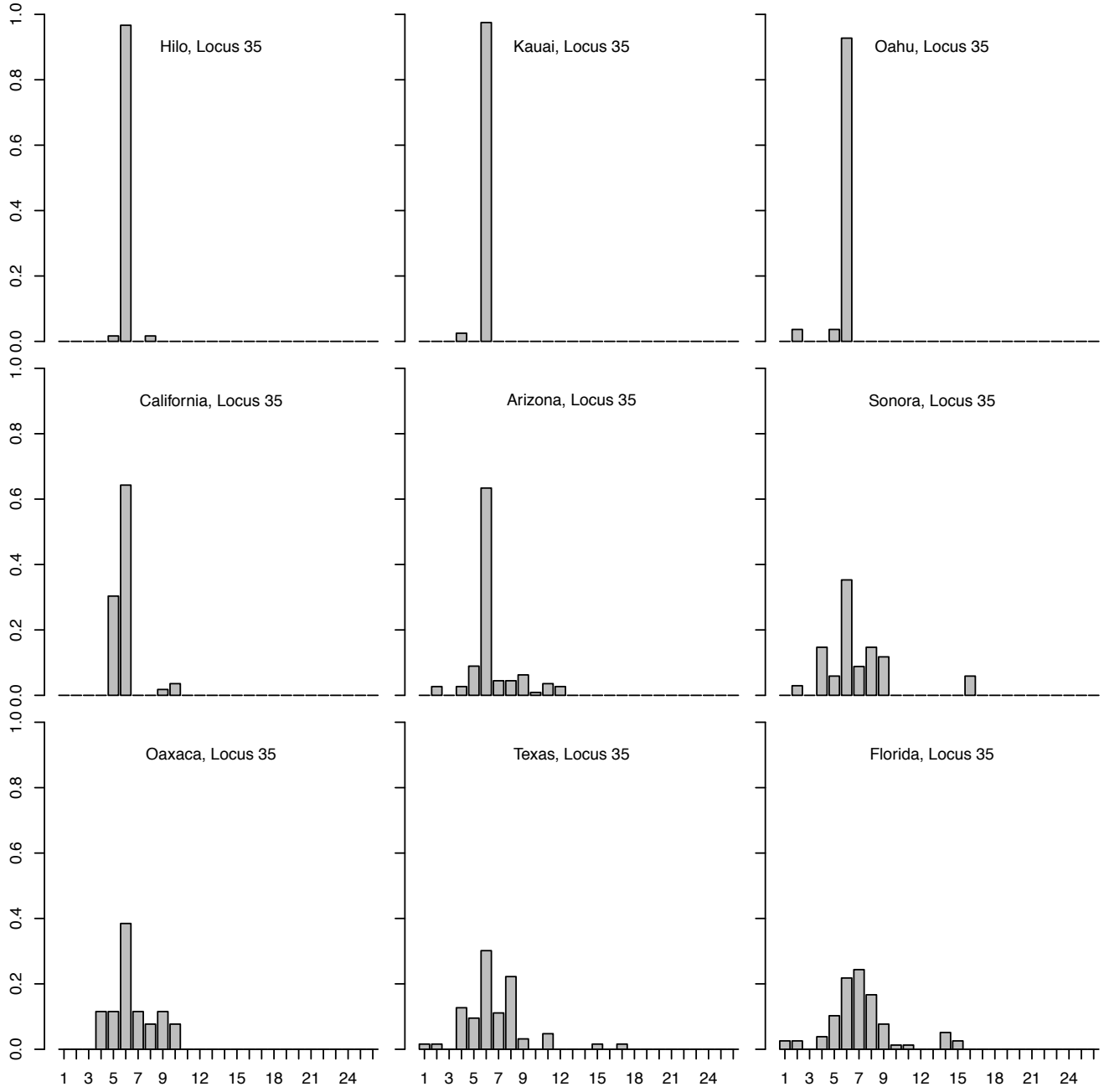


Figure 3. Allele frequency histograms for msat locus 35 for each population.

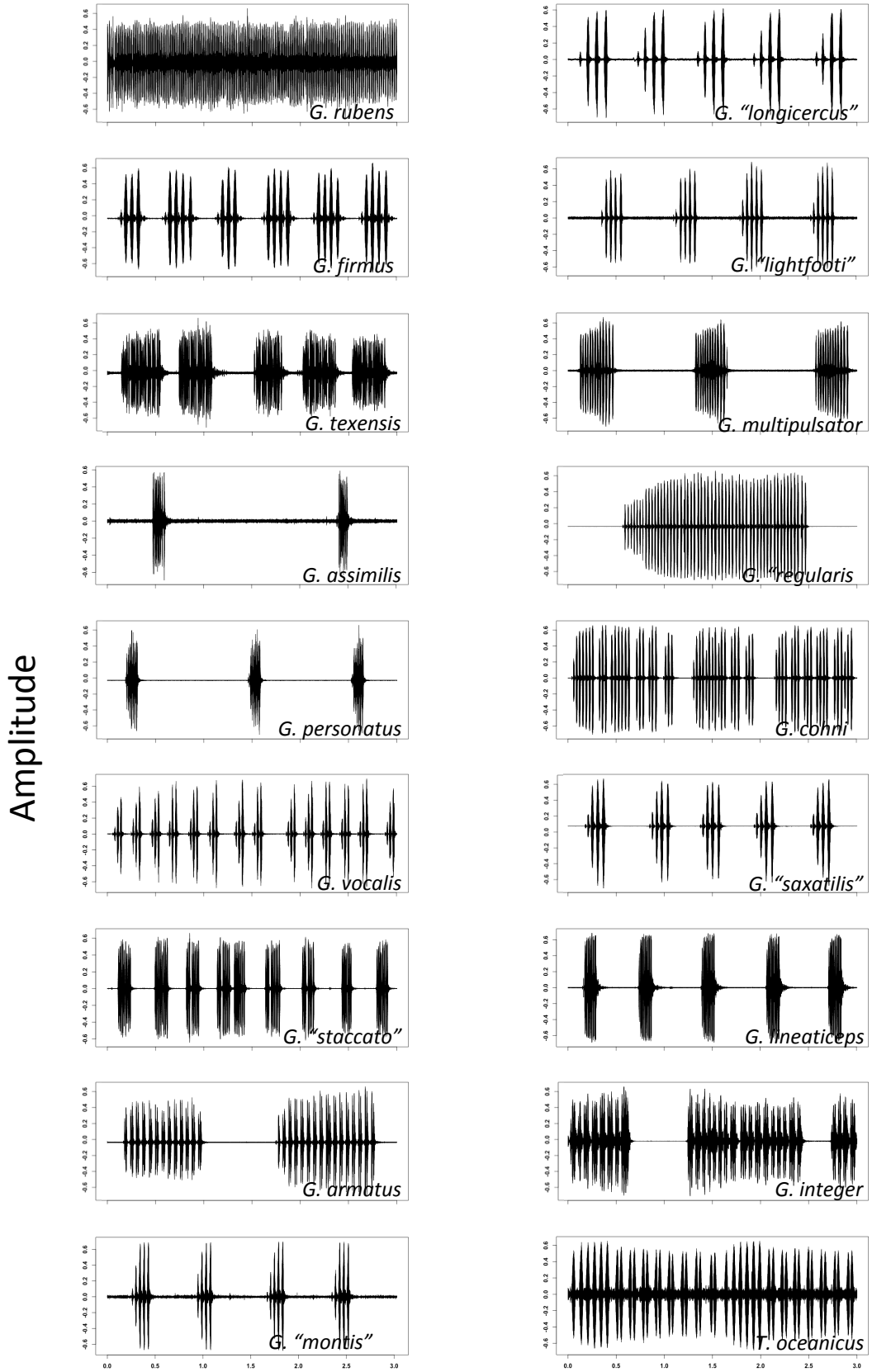


Figure 4. Waveform oscillograms of 3 seconds of song from confirmed host species showing overall song structure (chirps/trills).

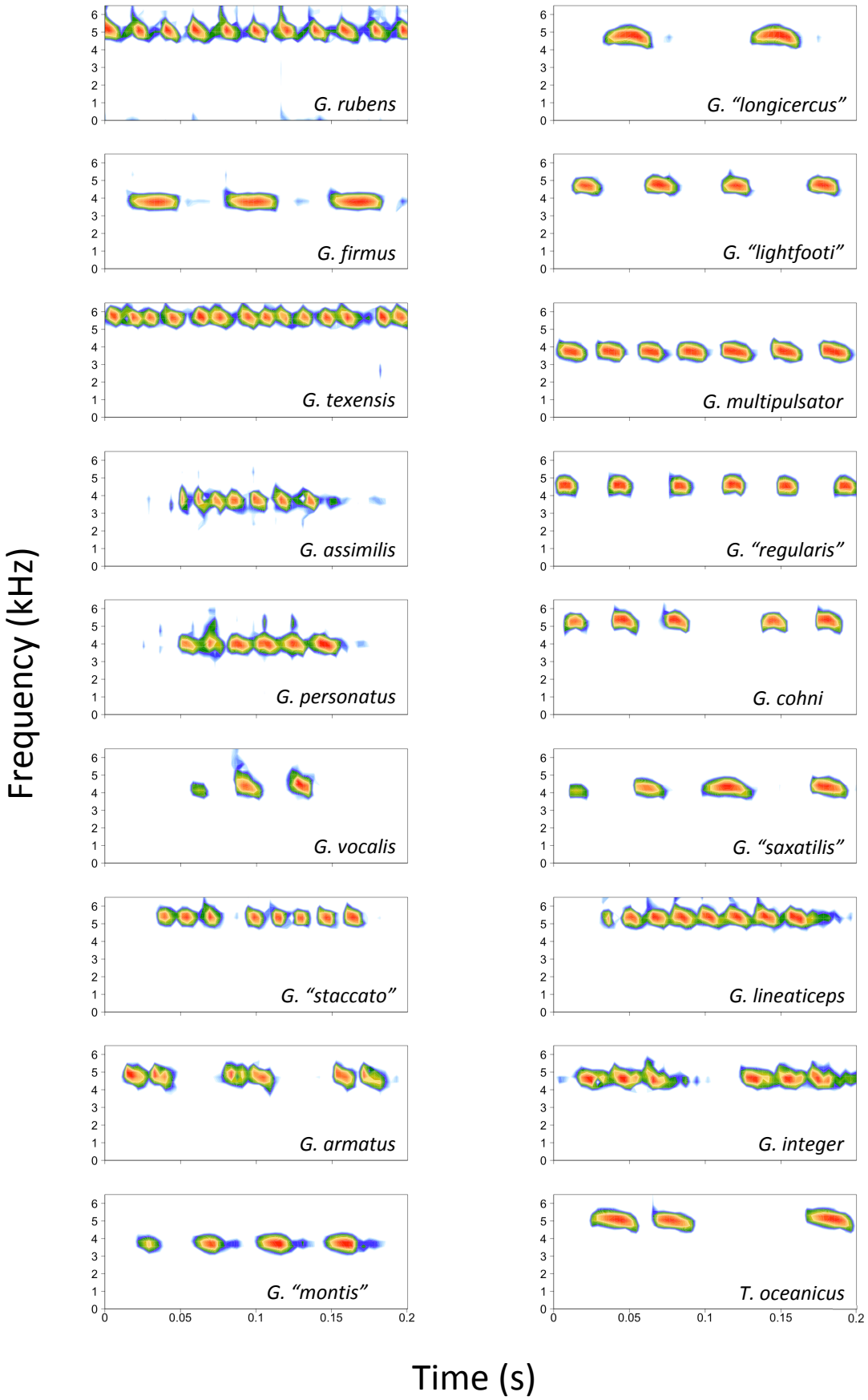
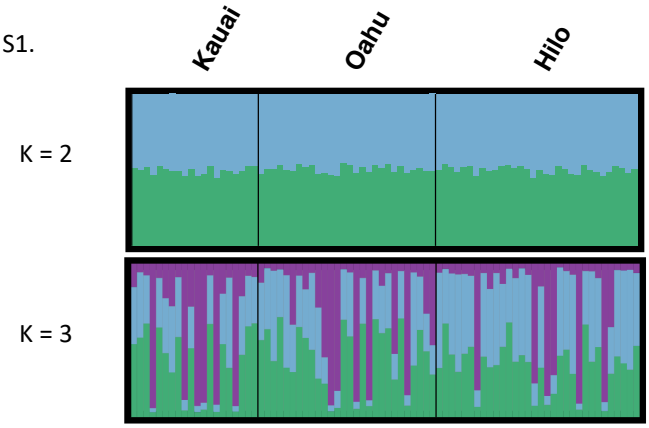


Figure 5. Spectrogram representations of 0.2 seconds of song from confirmed host species showing fine-scale song structure (pulses).

	G. rubens	*G. firmus*	*G. texensis*	*G. assimilis*	*G. personatus*	*G. vocalis*	*G. "staccato"*	*G. armatus*	*G. "montis"*	*G. "longicercus"*	*G. "lightfooti"*	*G. multipulsator*	*G. "regularis"*	*G. cohni*	*G. "saxatilis"*	*G. lineaticeps*	*G. integer*
G. firmus	4.01																
G. texensis	2.21	4.46															
G. assimilis	3.90	3.36	3.79														
G. personatus	3.79	2.62	3.49	1.76													
G. vocalis	3.91	1.68	3.96	3.70	3.30												
G. "staccato"	3.27	3.35	2.33	3.36	3.22	2.22											
G. armatus	5.44	4.21	5.17	3.63	3.33	4.82	5.08										
G. "montis"	4.29	1.15	5.01	3.19	3.00	2.23	3.87	4.28									
G. "longicercus"	4.04	1.41	4.74	4.22	3.80	1.38	3.40	5.18	1.69								
G. "lightfooti"	3.84	1.40	4.47	3.88	3.58	1.10	3.05	5.02	1.61	0.44							
G. multipulsator	2.91	2.74	2.89	1.20	1.38	2.99	2.56	3.84	2.86	3.51	3.19						
G. "regularis"	1.50	2.88	3.02	3.64	3.52	2.86	3.09	4.97	3.04	2.71	2.54	2.70					
G. cohni	3.74	2.61	3.75	4.30	3.65	2.38	3.23	3.89	3.18	2.70	2.63	3.60	2.80				
G. "saxatilis"	4.01	0.28	4.47	3.14	2.47	1.78	3.37	4.07	0.97	1.56	1.50	2.58	2.88	2.70			
G. lineaticeps	3.58	3.18	2.39	3.47	2.38	2.85	1.96	4.56	4.05	3.82	3.61	2.57	3.58	3.25	3.23		
G. integer	5.61	5.15	5.24	5.17	4.97	5.06	5.05	3.05	5.38	5.52	5.36	4.97	5.26	4.14	5.12	5.02	
T. oceanicus	8.24	7.61	7.93	8.17	7.57	7.79	8.02	7.07	7.97	8.02	8.00	7.91	7.95	6.86	7.63	7.52	7.52

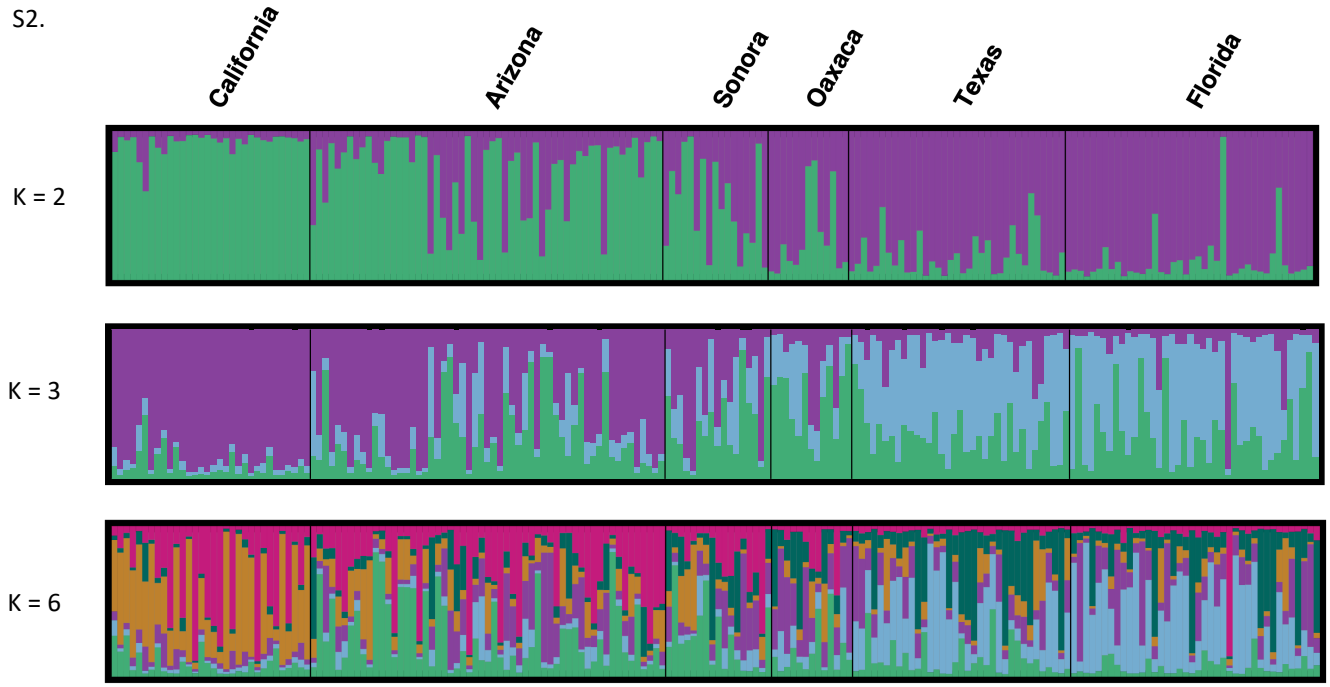
Figure 6. Euclidean pairwise inter-host song distances with heatmap colors indicating similar songs (green) or strongly divergent songs (red).

S1.

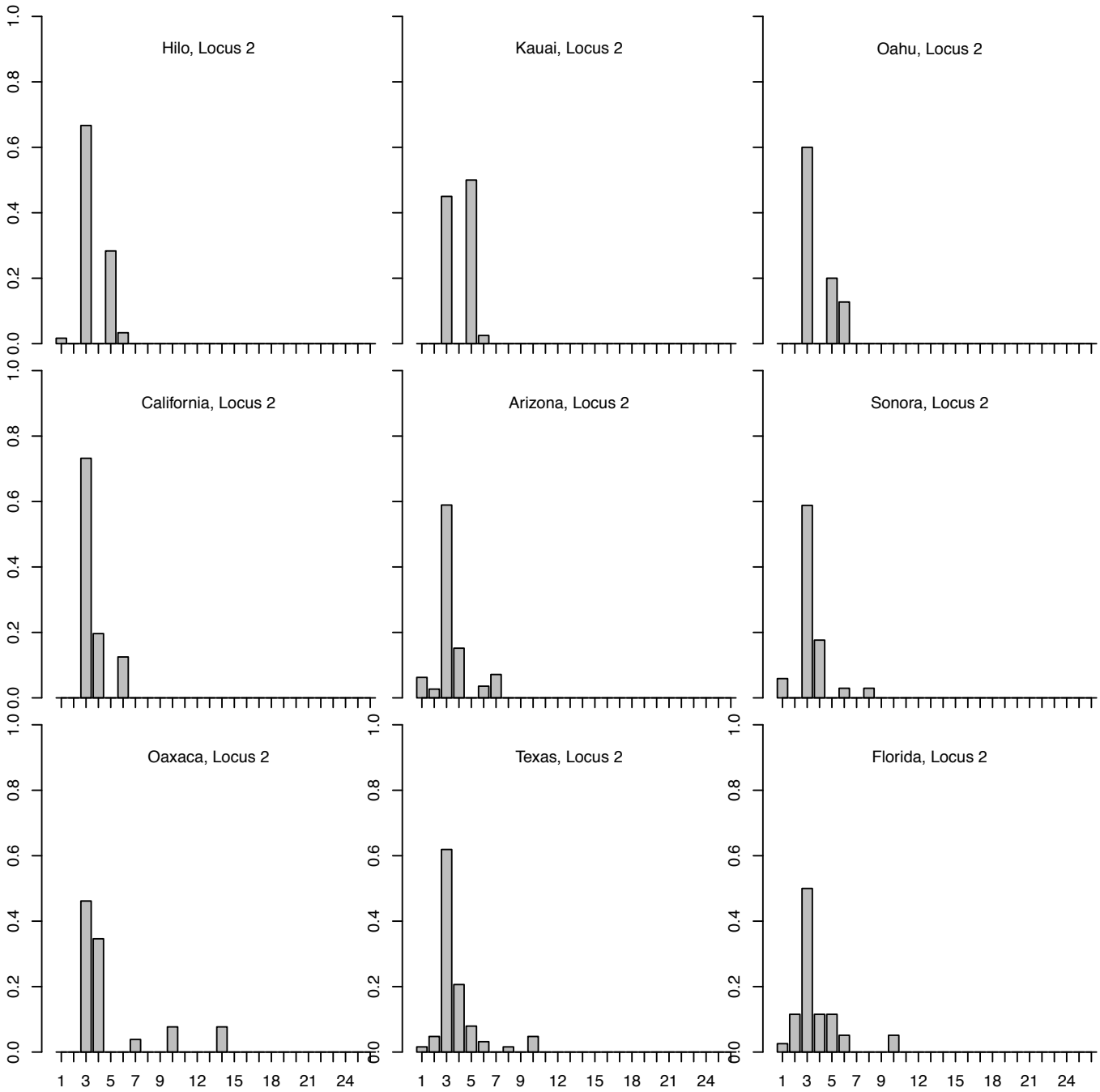


Supplemental Figure S1. STRUCTURE plots for Hawaii flies (K=2 and K=3)

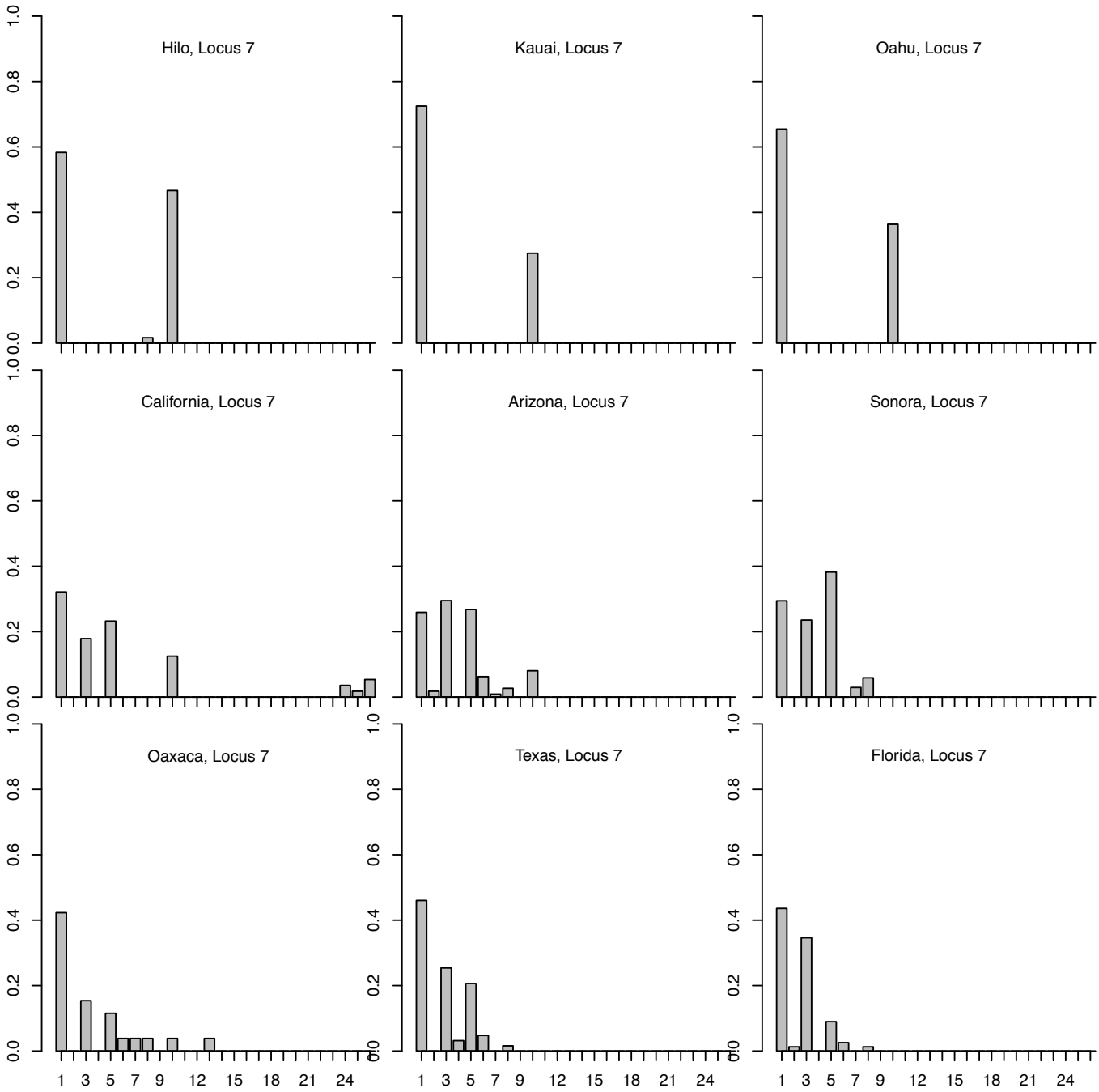
S2.



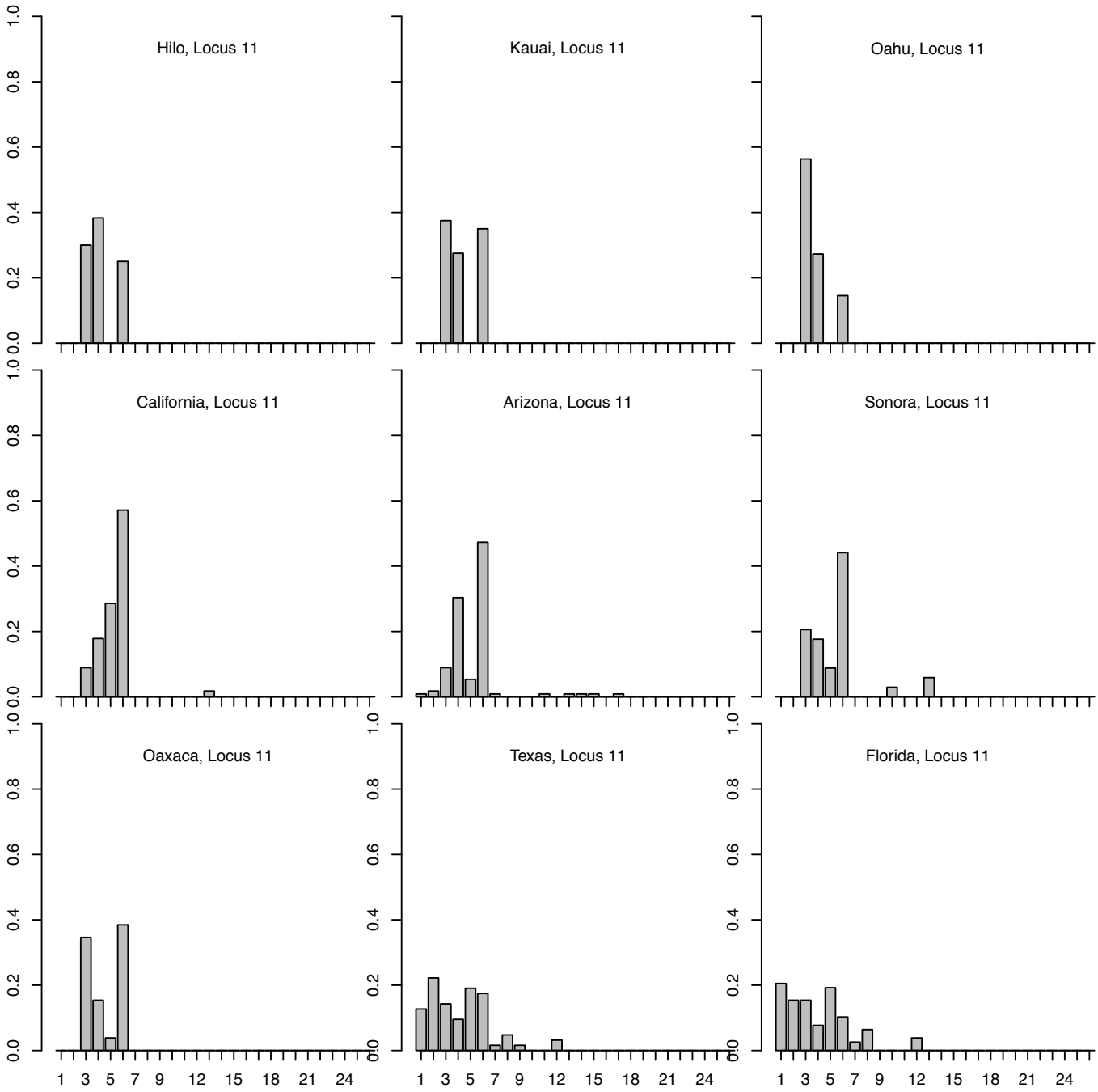
Supplemental Figure S2. STRUCTURE plots for mainland flies (K=2, K=3, and K=6)



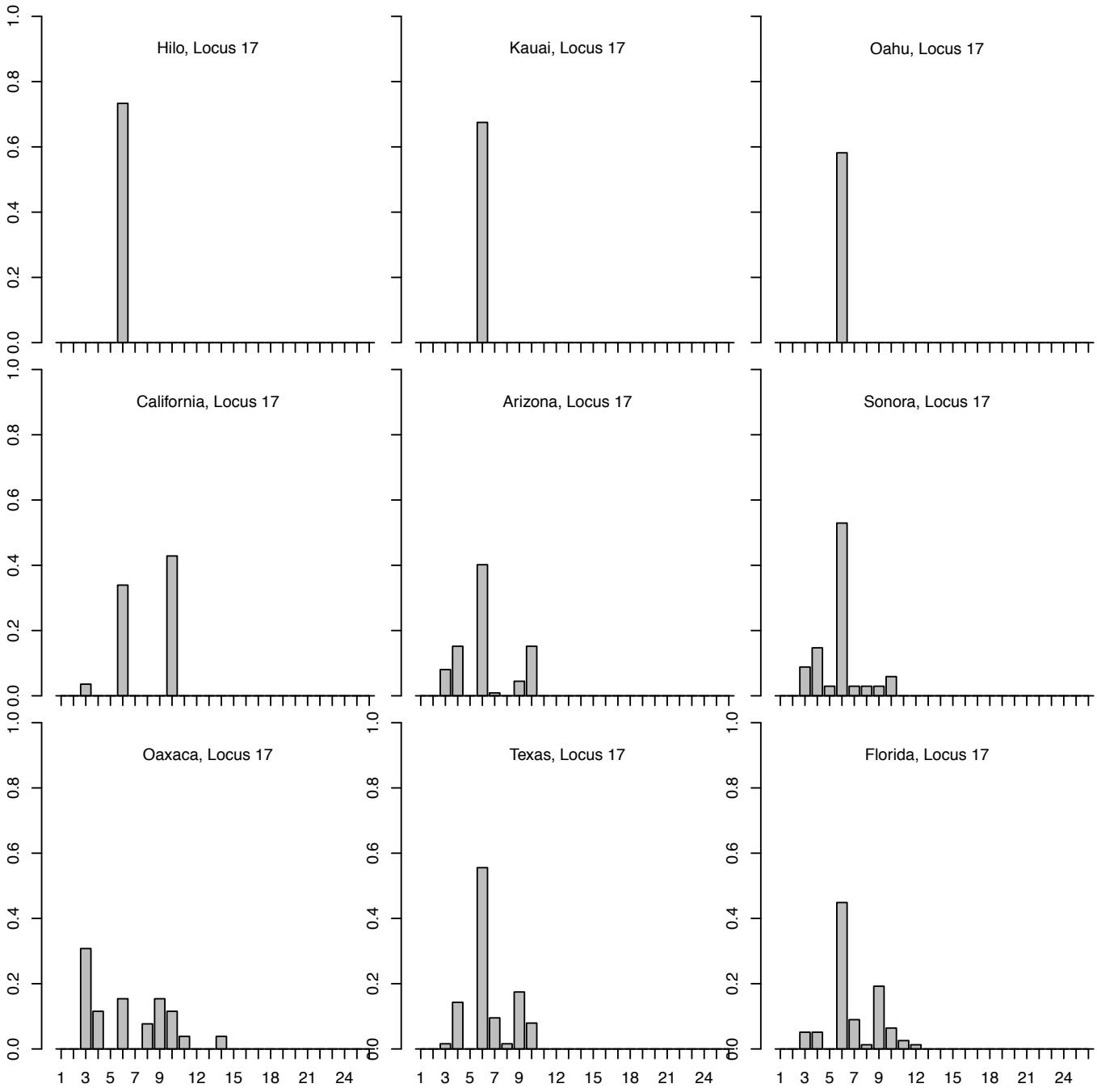
Supplemental Figure S3. Allele frequency histograms for msat locus 2 for each population.



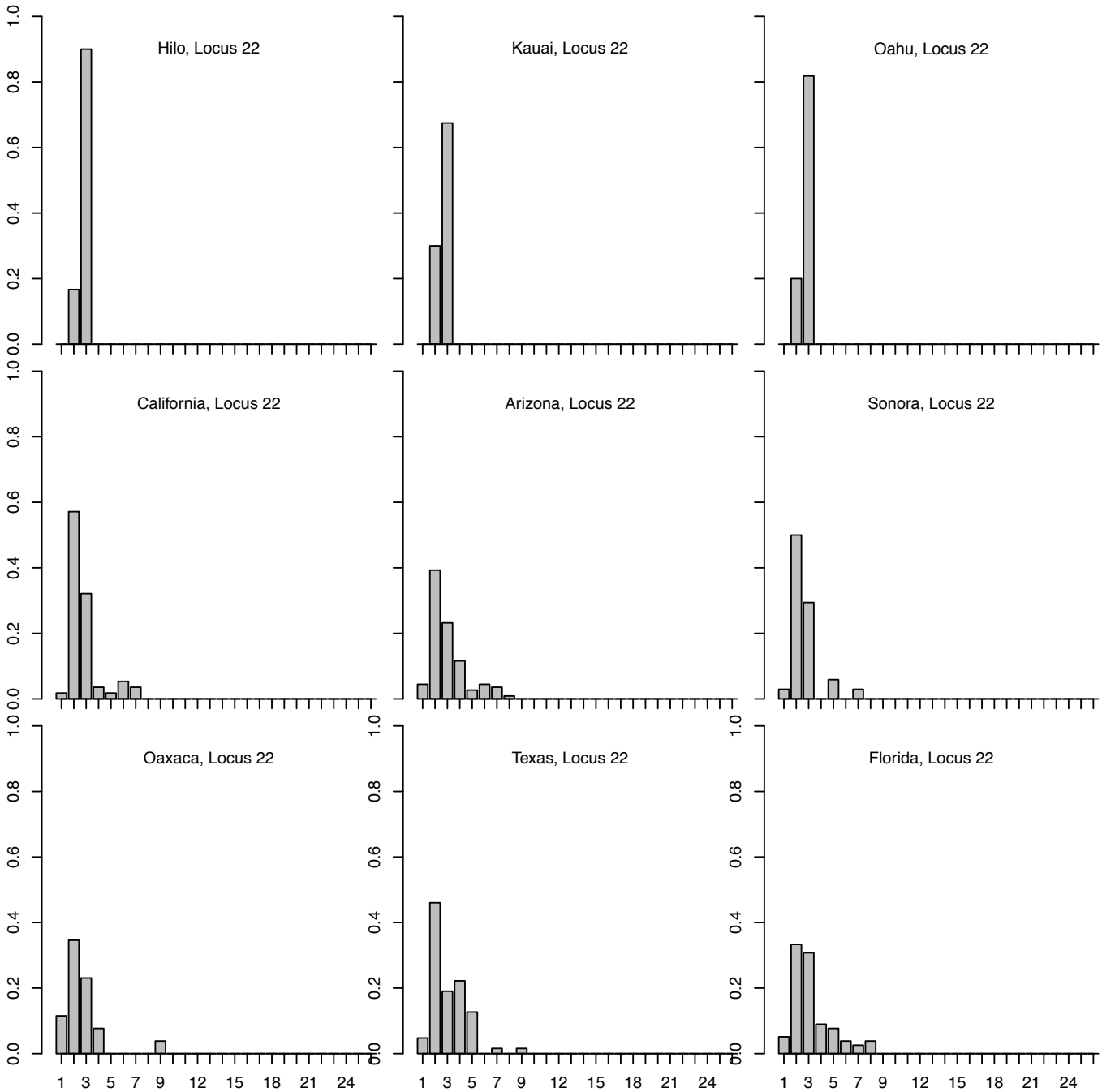
Supplemental Figure S4. Allele frequency histograms for msat locus 7 for each population.



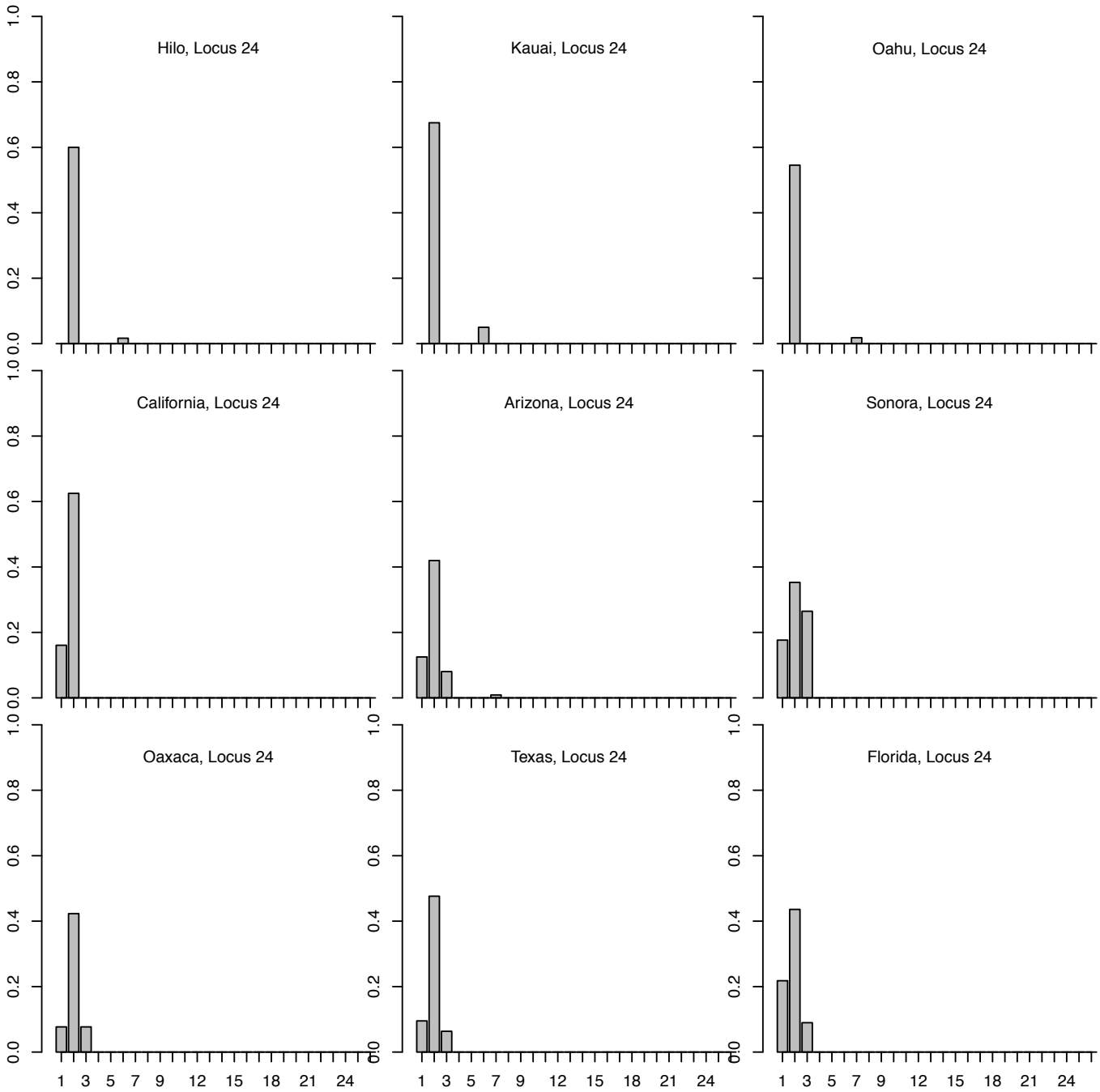
Supplemental Figure S5. Allele frequency histograms for msat locus 11 for each population.



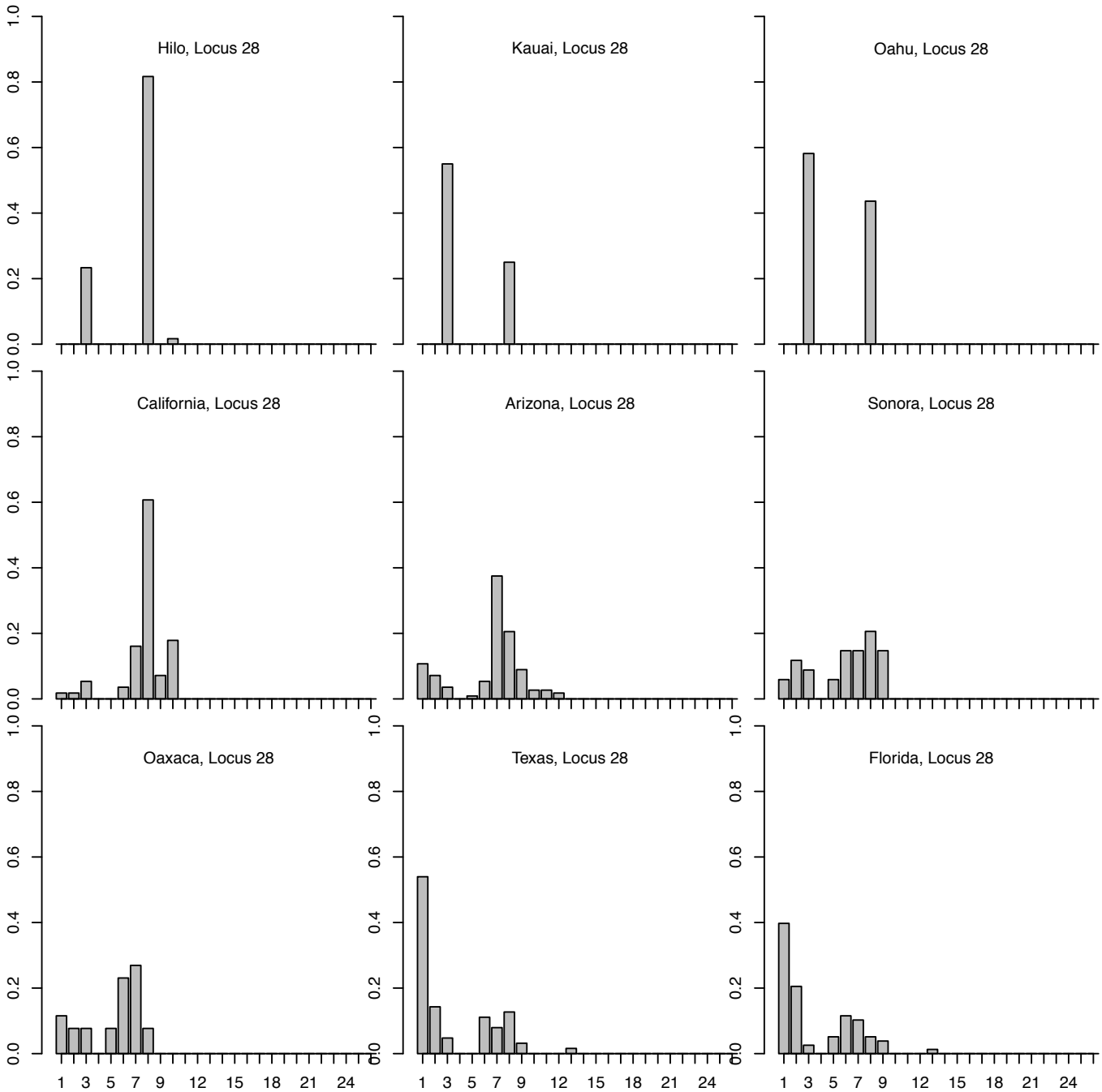
Supplemental Figure S6. Allele frequency histograms for msat locus 17 for each population.



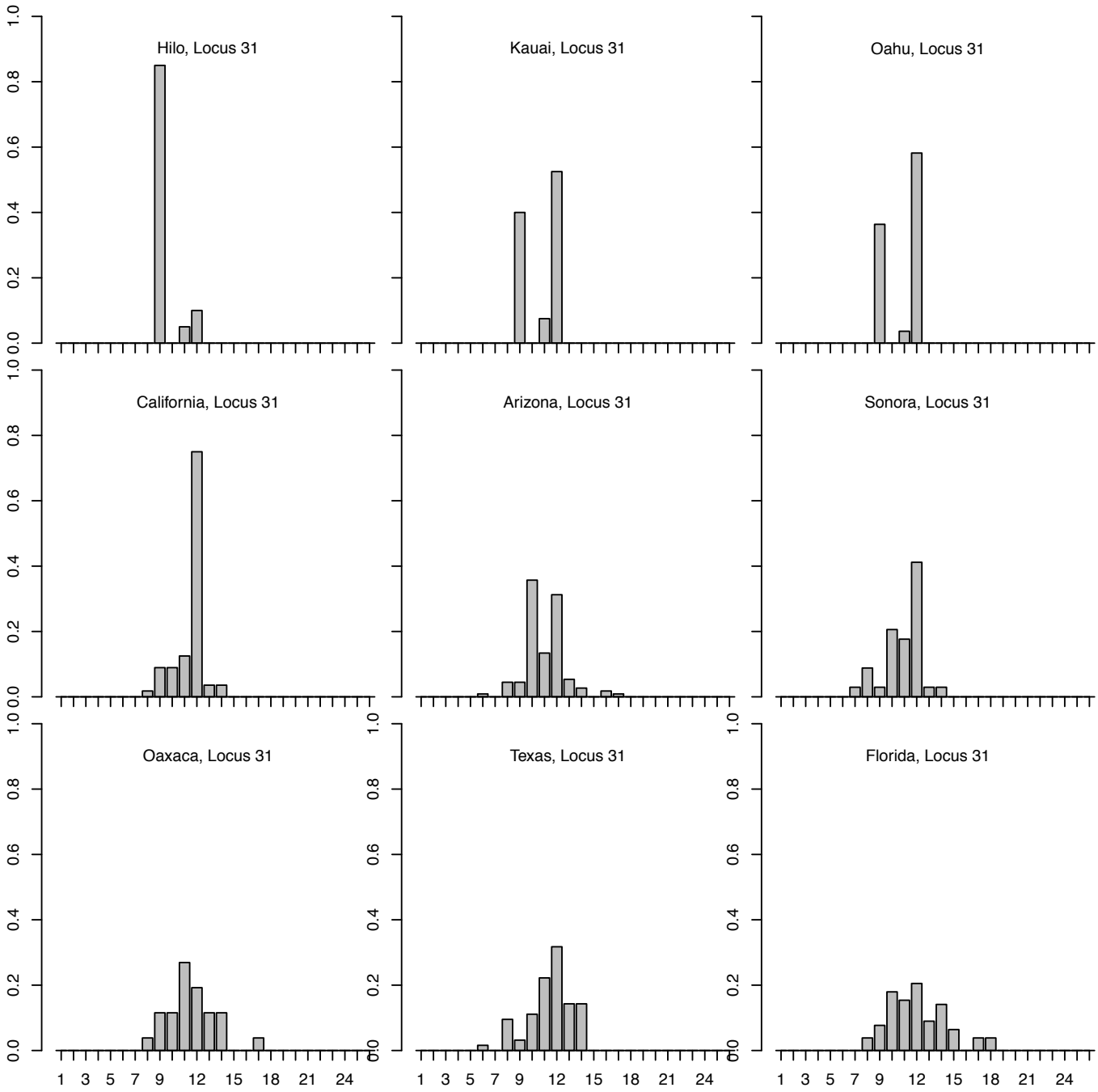
Supplemental Figure S7. Allele frequency histograms for msat locus 22 for each population.



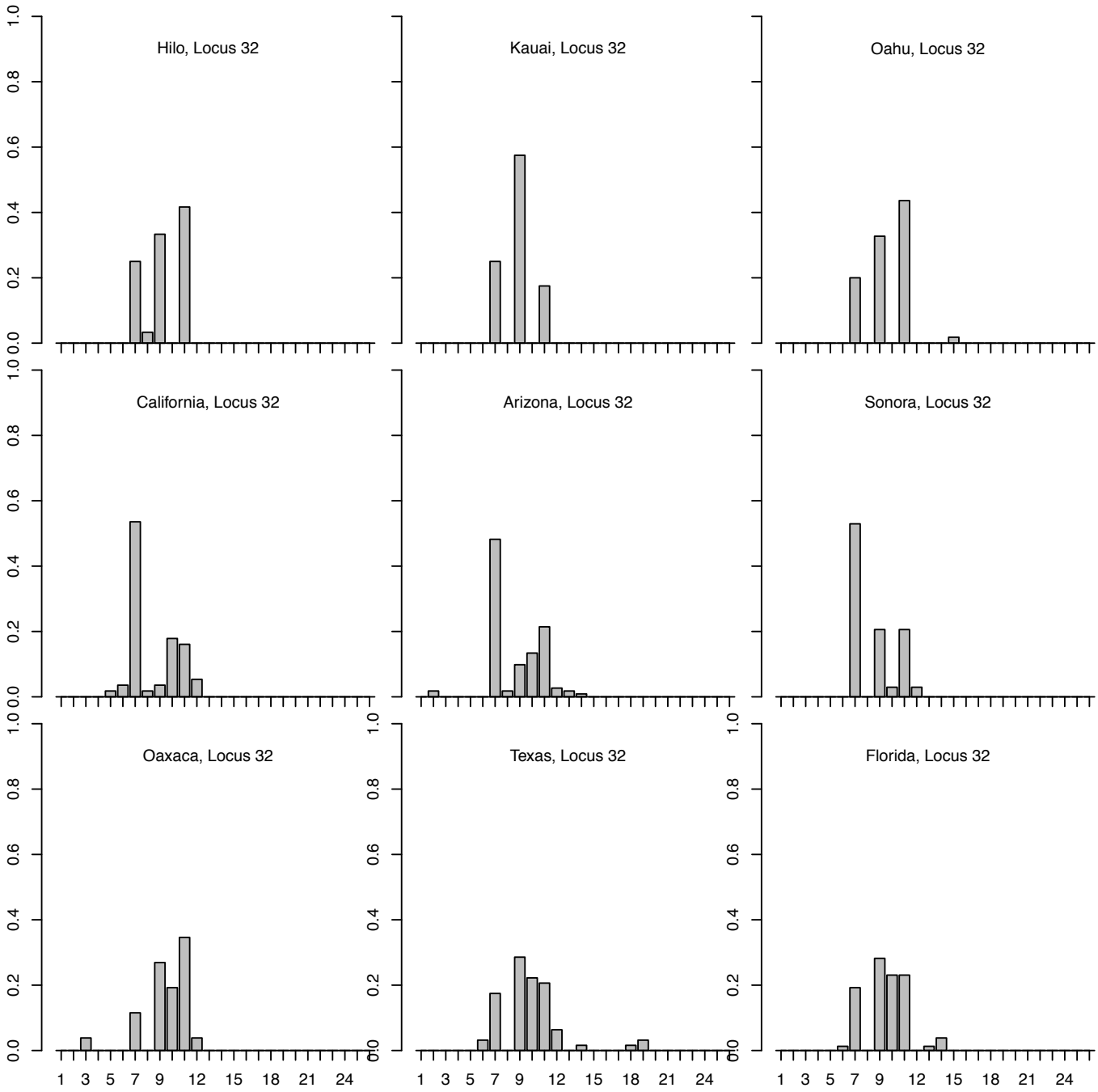
Supplemental Figure S8. Allele frequency histograms for msat locus 24 for each population.



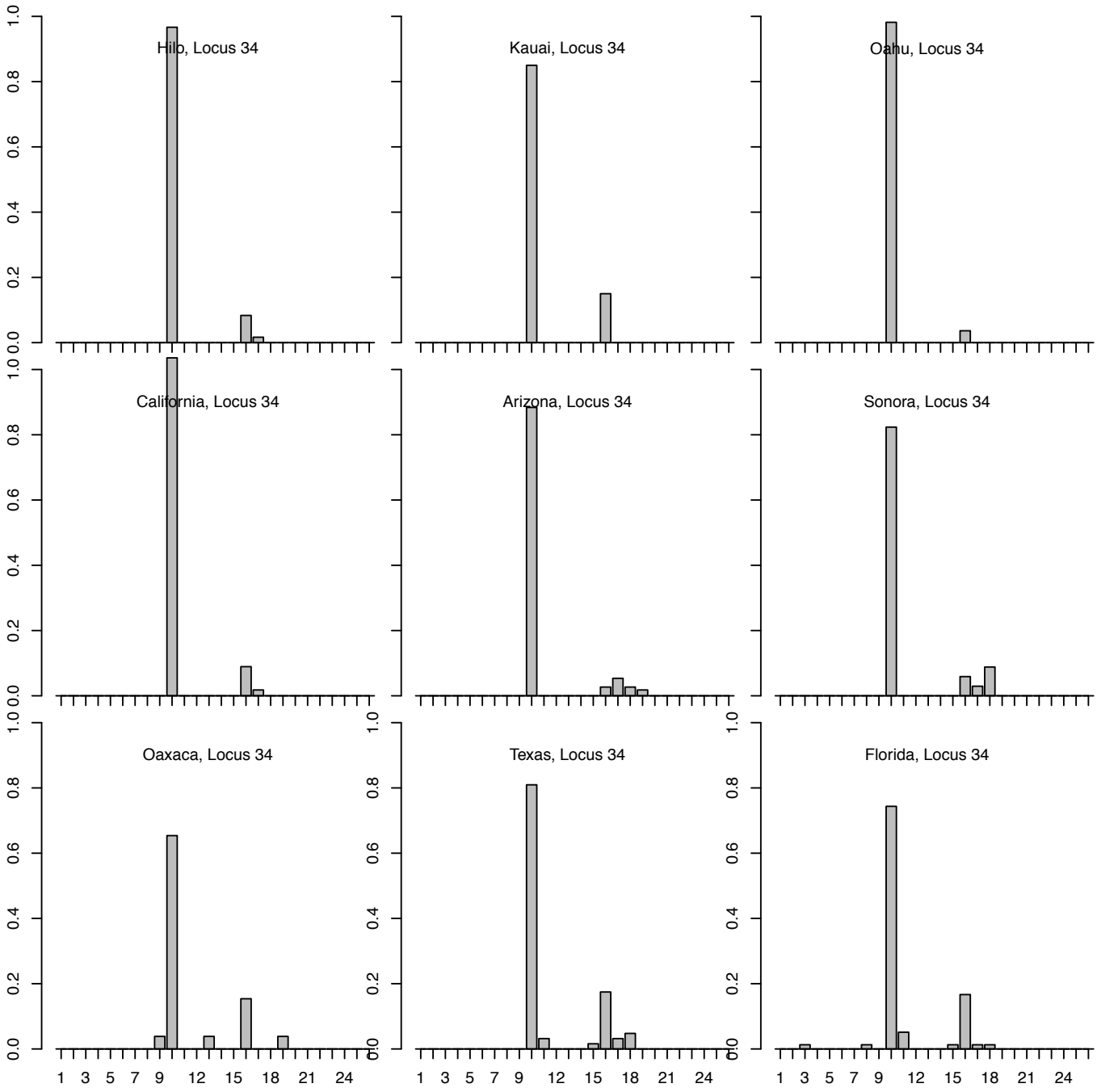
Supplemental Figure S9. Allele frequency histograms for msat locus 28 for each population.



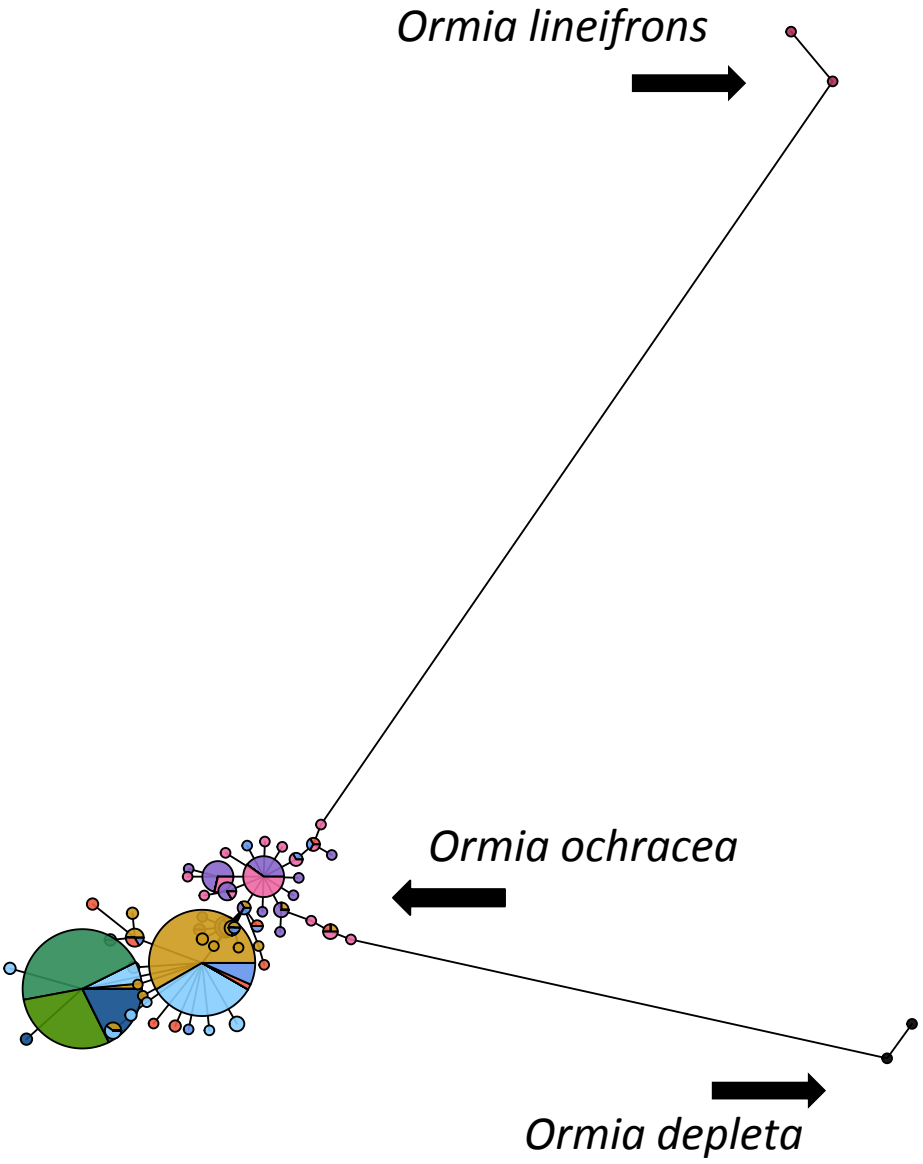
Supplemental Figure S10. Allele frequency histograms for msat locus 31 for each population.



Supplemental Figure S11. Allele frequency histograms for msat locus 32 for each population.



Supplemental Figure S12. Allele frequency histograms for msat locus 34 for each population.



Supplemental Figure S13. *Ormia ochracea* haplotype network with outgroups *O. lineifrons* and *O. depleta* appended.