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Original Article

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Sexual signal divergence and reproductive isolation in an adaptive radiation of a Hawaiian spider

Seira A. Adams[1,2,3,*], Jennifer Lee[3], Freddy Gutierrez[3], Stefan Schulz[4], Rosemary G. Gillespie[3], Santiago R. Ramirez[1,2]

[1]Center for Population Biology, University of California-Davis, Davis, CA 95616, United States
[2]Department of Evolution and Ecology, University of California-Davis, Davis, CA 95616, United States
[3]Department of Environmental Science, Policy, and Management, University of California-Berkeley, Berkeley, CA 94720, United States
[4]Institute of Organic Chemistry, Technische Universität Braunschweig, Braunschweig 38106, Germany

*Corresponding author. Center for Population Biology, University of California-Davis, 2320 Storer Hall, Davis, CA 95616, United States. E-mail: saadams@ucdavis.edu

Abstract

Adaptive radiations have long served as key models for understanding the process of speciation, yet the relative timing and role of ecological vs. sexual divergence in the rapid formation of reproductive isolation remains unclear. Many of the sexual signalling traits shown to play a key role in adaptive radiations are deeply intertwined with the ecological factors that power the ecological divergence and hence there is still much uncertainty as to how these sexual traits themselves drive diversification. Using a combination of chemical, behavioural, and genetic data, this study investigates the role of chemical sexual signals in *Tetragnatha quasimodo*, a species within an adaptive radiation of Hawaiian spiders, where populations are genetically divergent yet maintain similar niches. We find that the chemical signals of these spiders vary substantially with four distinct chemical profiles (chemotypes) that were not associated with measured environmental factors nor genetic distance. Behavioural trials showed strong assortative mate preference with males significantly choosing the same chemotype as themselves over other different chemotypes. There was substantial genetic differentiation between chemotypes, and individuals of a specific chemotype were more closely related to different chemotypes on the same island than to the same chemotype on different islands. Lastly, on the youngest island (Hawai'i), chemical differences were found in populations that were genetically similar, suggesting the possibility that sexual signal divergence can evolve before genetic divergence. These findings provide key insights into the role chemical sexual signals play in the attainment and maintenance of reproductive barriers in isolation of the ecological divergence found in this lineage.

Keywords speciation, sexual selection, pheromones, lipids, methyl ethers, Tetragnathidae

Introduction

Adaptive radiations have played a crucial role in developing our understanding of evolution as a whole (Schluter 2008, Grant and Grant 2014, Gillespie *et al.* 2020). Especially important is their role in understanding the mechanisms of speciation and how reproductive isolation is attained and maintained between taxa. Ecological speciation—the divergence due to adaptation to distinct ecological pressures—has classically been viewed as an important driver in the early stages of speciation (Losos and Mahler 2010, Stroud and Losos 2016). For example, the 'stages' model of adaptive radiation focuses on the role of the environment and ecology in the initial stages of reproductive isolation (Todd Streelman and Danley 2003). However, growing evidence suggests that in many taxa, initial

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reproductive isolation might be achieved with little ecological differentiation and under similar ecological pressures (Anderson and Weir 2020, 2022). While neutral processes such as drift and mutation-order speciation have been proposed for initiating reproductive isolation under such conditions (Schluter 2009), these mechanisms are generally slow (Coyne and Orr 2004, Lynch *et al.* 2016), leading to the question of how barriers to gene flow can initially form in rapidly splitting lineages of adaptive radiations.

In many adaptive radiations, premating sexual signalling traits have been shown to play a key role in initiating reproductive isolation (Gillespie *et al.* 2020). Nevertheless, there is still much uncertainty as to whether and how sexual signalling traits themselves drive diversification, as many of these sexual traits are deeply intertwined with the ecological factors that power the ecological divergence (e.g. the changes in beak morphology associated with both the diet and mating song in Darwin's finches) (Boughman 2001, Podos 2001, Seehausen *et al.* 2008, Maan *et al.* 2017). Specifically, there is much to be learned about the timing of sexual isolation relative to ecological shifts in the course of speciation, and how each contributes to the persistence of populations and lineages over time. To understand the relative roles and timing of divergence based on ecological traits and sexual signalling traits in the formation of species, we need a system in which processes can be teased out in time. The current study focuses on a single species within an adaptive radiation of Hawaiian spiders (long jawed orb-weavers, genus *Tetragnatha*) where populations appear to be genetically divergent, yet maintain similar niche space (Roderick *et al.* 2012). By investigating the chemical mating signals of these spiders across a temporal gradient, represented by the chronological formation of the Hawaiian Islands, we can measure how the sexual signalling traits are modified through space and time while keeping ecological traits constant.

The Hawaiian Islands offer natural experiments for studying the mechanisms of diversification as the linear chronosequence of islands creates snapshots of the evolutionary process over time, from 0 to 5 Myr (Clague and Sherrod 2014, Shaw and Gillespie 2016, Lim and Marshall 2017). Members of the Hawaiian *Tetragnatha* radiation are nocturnally active predators, forming a clade of over 50 species, most of which are endemic to single islands or volcanoes within the archipelago (Gillespie 1991, 1992, 2002, 2016). While known outside Hawai'i for their relative consistency in morphology (drab coloration, elongated body) and ecology (propensity to build horizontal orb webs over water), *Tetragnatha* spiders in Hawai'i, though occurring in a single habitat type (the high-elevation wet forest), exhibit a wide array of morphologies and ecologies (Hiller *et al.* 2019). The Hawaiian radiation is divided into two major clades, 'web-building' and 'spiny leg', the latter comprising species that have abandoned web-building and live a cursorial lifestyle (Gillespie 1991, 1992, 2002, Kennedy *et al.* 2022). Members of the spiny leg clade have evolved into four ecologically distinct niche specialists termed 'ecomorphs', with one member of each ecomorph generally co-occurring at any given site (Gillespie 1991, 2002, 2004, Kennedy *et al.* 2022). These ecomorphs are easily distinguishable by their body coloration and behaviour, with maroon ecomorphs preferring mosses, green preferring leaves, and small and large brown ecomorphs preferring twigs and branches respectively. Most of the members in the spiny leg clade show repeated evolution of ecomorphs, with the major exception being the 'large brown' ecomorph, represented by *T. pilosa* on Kaua'i, and the single species *T. quasimodo* that is found on all the other high islands except Kaua'i. Previous work on the Hawaiian *Tetragnatha* spiders has shown that species recognition is probably achieved through chemicals on the silk and body (Adams *et al.* 2024). These chemicals are long-chain alkyl methyl ethers and each species has a unique combination of methyl ethers that form a species-specific profile (Adams *et al.* 2024). Unlike many other spiders, *Tetragnatha* lack a complicated mating ritual, and the entire mating process consists of males locating a female by following her silk thread, then immediately approaching her, locking jaws, and mating (Levi 1981, Danielson-François *et al.* 2002). Male Hawaiian *Tetragnatha* spiders appear to use the species-specific combinations of methyl ethers found on the silk and body of the females to distinguish conspecific from co-occurring heterospecific mates. From this we can hypothesize that methyl ethers may play a key role in population divergence and reproductive isolation.

In this study we combine genetic, chemical, and behavioural data to examine how the chemical sexual signalling traits of *T. quasimodo* have evolved across the archipelago and how they relate to the genetic divergence across populations. If sexual signalling traits drive the initial diversification, then the divergence in chemical signals will be associated with divergent preferences and strong preferences for local signals, with signal differences preceding genetic differences. On the other hand, if the geographical landscape drives the diversification, then the divergence in chemicals and/or preference will increase linearly with geographical distance (or time in allopatry) and both the chemical differences and genetic differences seen across populations will be associated with island/substrate age. To address these hypotheses, we collected chemical data from 350 individuals, behavioural data from 125 trials, and genetic data from 113 individuals across populations from different islands and substrate age. We found that *T. quasimodo* has four distinct chemical profiles termed 'chemotypes'; each chemotype is composed of a single structurally unique methyl ether. These chemotypes were not associated with any broad-scale ecological factors known to commonly influence chemical differences in other arthropods, nor were they associated with geographical distance between populations. Male *T. quasimodo* significantly preferred the chemotype that matched their own chemotype and there was major genetic differentiation between the chemotypes found on each island, with evidence of chemical differences preceding genetic differences on the youngest island of Hawai'i. These results suggest that chemical recognition can evolve prior to genetic differentiation between populations.

Materials and methods

Study organisms and sites

Adult female, adult male, and juvenile spiders of *T. quasimodo* were collected by hand in Hawai'i during the years 2022–2024. Specimens collected for behavioural analysis were housed in modified 11 × 7 × 6-cm Tupperware containers with wet cotton balls for moisture and a plastic stick for shelter. They were maintained in an incubator set to a thermal cycle of 10–20°C and a 12-h light and 12-h dark cycle. The spiders were fed once a week with ca. 5–10 *Drosophila hydii* or *D. melanogaster* each. Specimens collected for chemical analysis were kept in snap cap vials until extractions were performed the day after collection. To quantify the climate at each collection site, temperature and rainfall data were obtained from The Hawai'i Climate Data Portal (Longman *et al.* 2024). One-way analysis of variance (ANOVA) was performed followed by a post-hoc Tukey's Honest Significant Difference (HSD) test in R v.4.4.0 (R Core Team 2020) to determine if the general climate conditions associated with each chemotype were significantly distinct.

Chemical analysis of spider extracts

To assess the chemical profile of *T. quasimodo* individuals, chemicals from the body of the spiders were extracted with pentane (≥ 99% purity) and analysed by gas chromatography-mass spectrometry (GC-MS, Agilent Technologies 7890B GC and 5977A MS). Specimens that were collected in individual snap cap vials were first killed in a −20°C freezer and then immediately transferred into a 2-mL glass vial containing 0.2 mL pentane. The legs were folded inward and the entire body was submerged in the solvent for 30 min before it was taken out and stored in 100% ethanol. The extracts and the specimens in ethanol were then stored at −20°C until further use. All tools were washed and cleaned using acetone, ethanol, and dichloromethane (DCM) before and after each step of the extraction. Before running on the GC-MS, all extract samples were fully evaporated and then 50 µL pentane mixed with an internal dodecane standard (1 µL D12: 100 mL DCM ratio) was added to the sample. The gas chromatograph was fitted with a 30 m × 0.25 mm × 0.25 µm HP-5 Ultra Inert column (Agilent Technologies) with helium as the carrier gas. Extracts were analysed in splitless mode, with a column oven temperature programme of 50°C for 5 min, increased by 5°C per minute to 320°C. Injector and transfer line temperatures were maintained at 250°C. GC-MS data were analysed in OpenChrom (Wenig and Odermatt 2010) by detecting chromatogram peaks using the First Derivative Peak Detector feature at the highest threshold. Peak integration areas and linear retention indices were calculated using the Trapezoid Peak Integrator and Retention Index Calculator features respectively. All integrated peak areas were visually inspected and corrected by manual integration if necessary. Previous work showed that methyl ethers are the most abundant compound found in the *Tetragnatha* spider silk and body extracts and are used in species recognition (Adams *et al.* 2021, 2024). As such, in this study, peaks with a strong *m/z* 45 were identified as methyl ethers (Schulz and Toft 1993) and cross-referenced to a user-built methyl ether library. Methyl ether peaks were aligned manually, based on retention time, retention index, and visual comparison with spectra in the library. For each individual chromatogram, relative peak contributions of each methyl ether were calculated by dividing the peak area of a specific methyl ether by the sum of the peak areas of all methyl ethers found in the sample.

Orbitrap ion series spectra

A Thermo Scientific Trace 1310 gas chromatograph (Thermo Scientific, Bremen, Germany) equipped with a 30-m analytical column (Phenomenex ZB5-MS, 30 m × 0.25 mm ID, t_f = 0.25 µm) was used in splitless mode with an injection temperature of 270°C. The temperature programme was: 100°C (3 min) increasing by 5°C per minute to 320°C (3 min). The helium carrier gas was set to a constant flow rate of 1.0 mL/min. The gas chromatograph was coupled to an Exactive GC-Orbitrap mass spectrometer (Thermo Scientific). The resolution was set to 60000 (FWHM; instrument setting at 200 u). The mass range was 40—500 u, and two micro scans were averaged per data scan. The automated gain control (AGC target) was set to 1×10^6, and the maximum inject time was set to 'auto'. The auxiliary temperatures were set to 290°C for transfer lines 1 and 2, and the electron ionization source temperature was set to 220°C. Electron ionization was performed at 70 eV energy in positive mode. Helium (carrier gas) and nitrogen (supply for the C-trap) were equipped with gas purification cartridges to trap moisture and organic impurities of the gases (Thermo Scientific). The C-trap energy offset for these measurements was 2.0 V. The column bleed ion at 207.03235 u was used as the lock mass for internal mass calibration of the data. The high-resolution spectra obtained were edited using Excel macros described in Kiuchi *et al.* (2025) to obtain spectra of the ion series spectra of $40 + (n \times 14)$ (ISS40) and $45 + (n \times 14)$ (ISS45).

Silk extract choice trials

To assess whether male *T. quasimodo* spiders could discriminate females with the same chemical profile from those with a different chemical profile, chemicals from the dragline silk of *T. quasimodo* females were extracted with DCM (analytical grade) and the extracts were presented to males on cotton threads in a silk extract choice trial. Previous work has shown that the methyl ether profiles found in the silk extracts are the same as those found in the body extracts (Adams *et al.* 2021). The same protocol described in Adams *et al.* (2024) was used for both the silk extraction and behavioural assays. Dragline silk naturally deposited inside the Tupperware enclosures was collected by wrapping the silk around a clean metal spatula and pushing it into a tight ball using clean tweezers. The silk ball was then placed in a 2-mL glass vial (Agilent Technologies), soaked in 0.2 mL DCM for 30 min and then removed from the vial with clean tweezers. The extracts were stored at −20°C until further use. In each trial, two pieces of cotton thread were attached to the end of a bamboo stick to form a Y-shape. The silk extracts were dispensed with a new glass pipette onto each cotton thread. After giving the extracts time to set for 1 min, a

male was placed at the opposite end of the bamboo stick facing the cotton threads with the extracts. In each trial, the male was given two extracts to choose from—the silk extract of a female from the same population as the male and the silk extract of a female from a different population. The male was considered to have made a choice when he moved off the bamboo stick and walked up one of the cotton threads. All trials were conducted in the dark under red light and chi-square analyses were performed to evaluate male choice. A total of 125 trials were conducted across nine different combinations of the two chemical profiles (females with either the 33A or 31A profile) and four collection sites (Upper Waikamoi in Maui as well as Kipuka, Hualālai, and Laupāhoehoe in Hawai'i). Chi-square (χ^2) goodness-of-fit tests were performed in R v.4.4.0 (R Core Team 2020) for each combination to determine statistical significance of the male choice.

Low-coverage whole genome resequencing

To assess the genetic structure of *T. quasimodo* across islands, populations, and chemotypes, the genomes of 113 *T. quasimodo* individuals that were chemically analysed were sequenced. For the outgroup, the genomes of eight *T. brevignatha* and one *T. waikamoi* (closely related Hawaiian species) were additionally sequenced. For each specimen, four legs from one side of the spider were pinched off at the base of the coxa using sterile tweezers, ground up using a tube pestle, and then incubated overnight in a lysis buffer solution and proteinase K (OMEGA Bio-Tek, Norcross, GA, USA) at 55°C. DNA was then extracted using solid-phase reversible immobilization (SPRI) beads following the Holmquist *et al.* (2025) protocol and eluted in 25–50 µL elution buffer. The concentration of each DNA extract was assessed using a Qubit fluorometer (ThermoFisher).

For library preparation, between 77 and 1281 ng of DNA from each sample was fragmented using a qSonica sonicator (Newton, CT, USA) until most material was between 300 and 500 bp. The samples were sonicated at 40% amplitude with a 10 s on/off pulse for 4 min 30 s. Samples that did not reach the target size were sonicated for an extra 1–2 min until the target size was reached. A double-sided SPRI bead cleaning process was used to refine the size-selection by removing fragmented DNA below 200 bp and above 600 bp. Dual-indexed libraries were prepped following a modified KAPA Hyper Prep (Roche) protocol (Benham *et al.* 2025) with dual indexing oligo sets custom-designed at the Functional Genomics Laboratory at UC Berkeley. The libraries were amplified with the KAPA HiFi 2× HotStart ReadyMix PCR Kit for 10–12 cycles. Libraries were then assessed for sizing on an agarose gel and Bioanalyzer DNA 1000 chip (Agilent Technologies). In total, 150 paired-end reads were sequenced on two lanes of the Illumina NovaSeq X 25B at the Vincent J. Coates Genomics Sequencing Lab (QB3 Genomics, UC Berkeley, Berkeley, CA, RRID: SCR_022170).

Bioinformatics data processing and analysis

High-quality variant calls for the *T. quasimodo* samples were generated using the snpArcher workflow (Mirchandani *et al.* 2024), a flexible workflow designed for the analysis of genomic resequencing data in nonmodel organisms. Using snpArcher, sequencing reads were first trimmed of adapters using fastp v.0.20.1 (Chen *et al.* 2018) and aligned to the *T. kauaiensis* reference genome (Cerca *et al.* 2021) using *bwa mem* v.0.7.17 (Li 2013) with default parameters. Individual variants were then called using GATK v.4.1.8.0 (McKenna *et al.* 2010) and a multisample variant call format (VCF) file was created. For quality control, sites that failed the set of standard GATK hard filtering thresholds defined in the snpArcher workflow were removed. For single nucleotide polymorphisms (SNPs), these criteria were QUAL < 30, QD < 2, FS > 60, SOR > 3, MQ < 40, MQRankSum < −12.5, and ReadPosRankSum < −8. Additionally, *bcftools* v.1.12 (Danecek *et al.* 2021) was used to remove individuals with < 2× sequencing depth, sites with minor allele frequency of < 0.01, and sites where more than 75% of the individuals lacked a high-confidence genotype call. Only biallelic SNPs were retained for downstream analysis.

To assess the evolutionary history of the *T. quasimodo* populations across the archipelago, a phylogenetic tree, a principal component analysis (PCA), and an admixture plot were constructed. To create the phylogenetic tree, the VCF file was first converted to a beagle file using Plink v.1.9 (Purcell *et al.* 2007). Then, ngsDist v.1.8.6 (Vieira *et al.* 2016), a software optimized to run trees on whole-genome low-coverage data, was used to create a pairwise genetic distance matrix specifying a block size of 20 SNPs and 100 bootstrap replicates. FastME v.2.1.6.3 (Lefort *et al.* 2015) was then used to build phylogenetic trees under the balanced minimum evolution method. Finally, RAxML-NG v.1.2.2 (Kozlov *et al.* 2019) was used to map the bootstrap values onto the tree. Trees were viewed and edited using FigTree v.1.4.4 (Rambaut 2009).

To assess the population genetic structure, PCA and ADMIXTURE were used. First, linkage disequilibrium (LD) pruning was conducted on the dataset using Plink v.2.0 (Chang *et al.* 2015) with a sliding window of 50 variants, a step size of 5, and an r^2 threshold of 0.1. Eigenvectors and eigenvalues were created using Plink v.1.9 (Purcell *et al.* 2007) and a PCA was graphed in R v.4.4.0 (R Core Team 2020). ADMIXTURE v.1.3.0 (Alexander *et al.* 2009) was run to test for genetic clustering with the most likely value of K (from K = 2–15) assessed using cross-validation error. Lastly, to assess the genetic divergence within and between islands, as well as within and between chemotypes, the nucleotide diversity (π), absolute genomic divergence (D_{xy}), and relative genomic divergence (F_{ST}) were calculated for all populations using Pixy v.2.0.0 (Korunes and Samuk 2021). Populations were defined by both the collection site and chemotype. Populations represented by only a single individual were excluded from the divergence analysis. Differences in pairwise D_{xy} and F_{ST} values across intra-/inter-island/chemotype combinations were assessed using a Kruskal–Wallis test followed by a post-hoc Dunn's test using the dunn.test package in R v.4.4.0 (Dinno and Dinno 2017, R Core Team 2020). All P-values were adjusted using the Benjamini–Hochberg (FDR) correction.

Results

Chemical profiles differed between populations of *T. quasimodo*

The chemical profiles of 350 individual *T. quasimodo* spiders from 42 populations across the Hawaiian archipelago were analysed using GC-MS. Of the 350 individuals chemically assessed, the chemical profile of *T. quasimodo* spiders almost always consisted of a single dominant methyl ether compound that had an average relative abundance of 98% in the profile (Figs 1, S1). The identity of this single dominant compound differed between individuals and was either 1-methoxy-2,12,18-trimethylheptacosane, 1-methoxy-8,14,20-trimethylnonacosane, 1-methoxy-2,14,20-trimethylnonacosane, or 1-methoxy-12,18,24-trimethyltritriacontane (hereafter 31A, 33A, 33B, or 37A respectively). The structure of these compounds was elucidated by combined analysis of their high-resolution Orbitrap spectra and gas chromatographic retention indices (Supplemental Results S1, Figs S2–S17, Tables S1–S8) (Kiuchi *et al.* 2025). The total number of methyl branches was consistent across all four chemotypes. The ω-methyl branch pattern provides a method to count the methyl branch position from the alkyl end of the ethers—the biosynthetic starting point of compounds derived from the fatty acid biosynthetic pathway in arthropods (Holze *et al.* 2021). Using this method, ethers 33A and 37A showed the exact same pattern of ω-9, ω-15, and ω-21 while ether 31A had ω-9, ω-15, and ω-25 and ether 33B had ω-9, ω-15, and ω-27 (Fig. 2). Of the 42 collection sites examined in this study, 38 were monotypic for a chemotype where all the individuals at that site had the exact same chemotype. The remaining four sites were sympatric for chemotypes and consisted of individuals with differing chemotypes (Figs 1, S18). When a mixture of chemotypes were found at a site, there were never more than two different chemotypes present (e.g. a mixture of individuals with either the 33A or 31A chemotype was found at a site in East Maui). The climate that each of the chemotypes inhabited across the archipelago did not differ significantly from one another (excluding 33B since it was sampled at only one site) (Fig. S19; Table S9). The annual mean air temperature and rainfall measured at the sites where 37A, 33A, and 31A were collected did not show a significant difference and were relatively comparable across sites.

Males preferred females with the same chemical profiles

A total of 125 silk extract choice trials were conducted to determine whether males can discriminate the silk extract of a female with the same chemical profile from those of a female with a different chemical profile. Males selected the silk extract of the female with the same chemical profile at a significantly higher frequency than the extract of the female with a different chemical profile (60 males out of the 75 tested chose the silk extract of the female with the same chemical

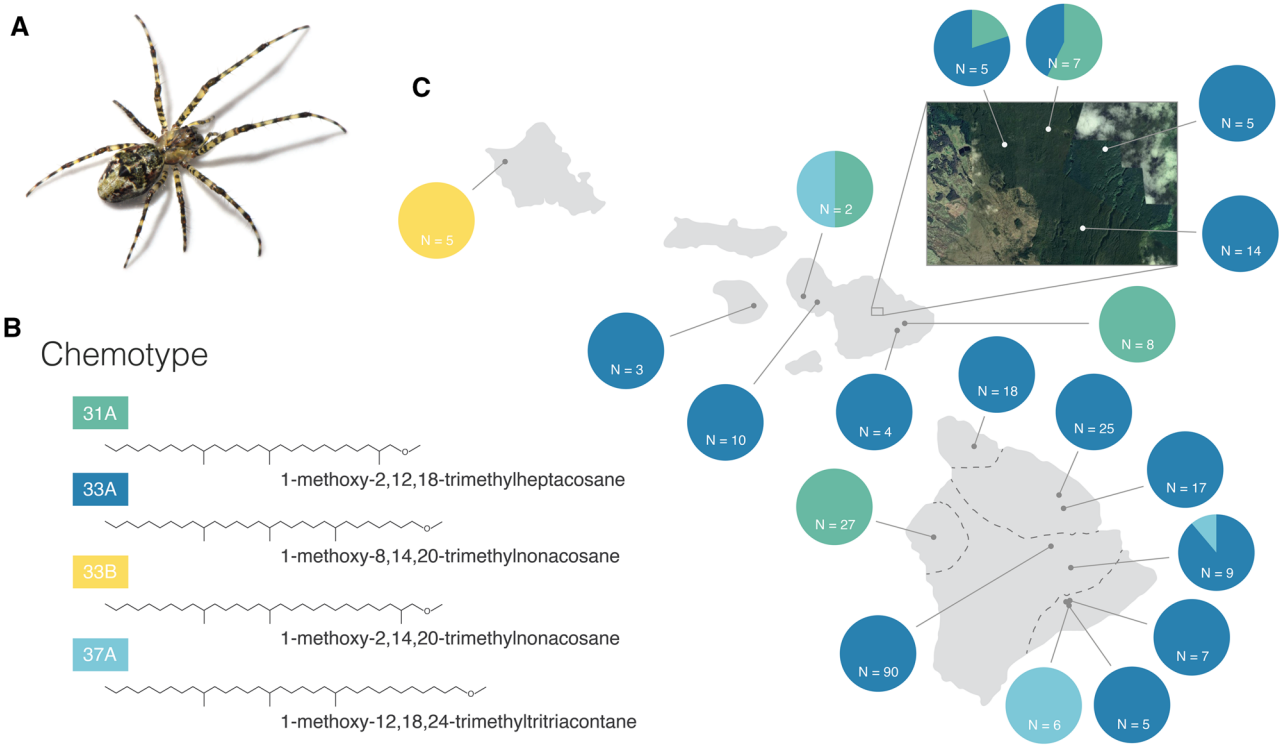

Figure 1 Chemotype variation across populations. The distribution of chemotypes across the Hawaiian archipelago. A, photograph of a T. quasimodo female. B, the four different chemotypes found in *T. quasimodo* and the identity and chemical structure of each methyl ether found in the chemotype. C, map showing the distribution of the chemotypes across the archipelago. Pie charts show the proportion of the chemotype found at each site. N, number of individuals. Imagery @2026 Airbus, CNES / Airbus, Data USGS, Landsat / Copernicus, Maxar Technologies, Map data @ 2026 Google.

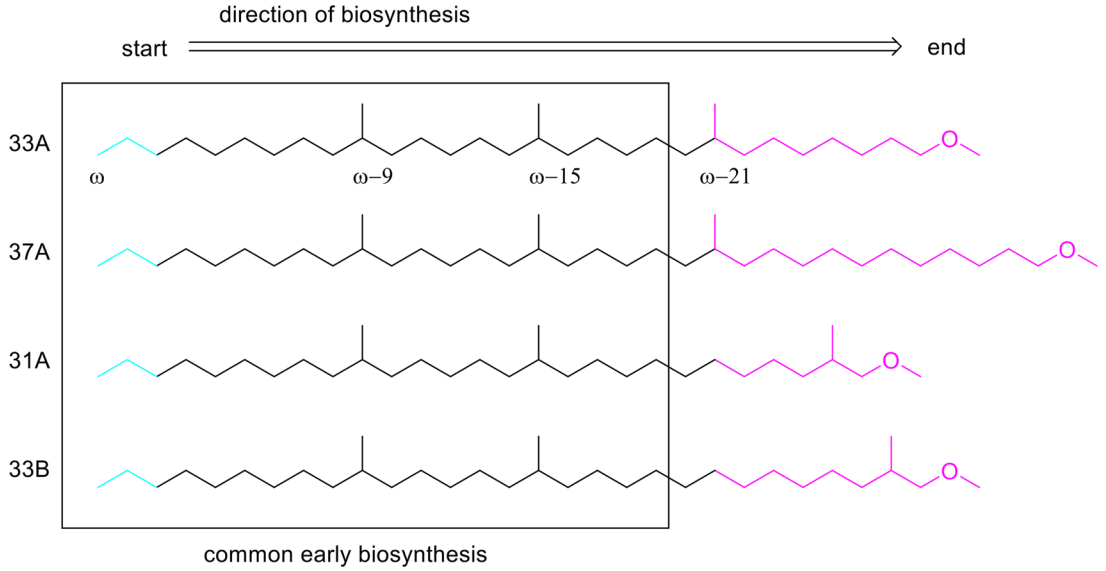

Figure 2 Proposed biosynthesis of the methyl ethers. The methyl ethers are aligned by the ω-end (the starting point of the biosynthesis of compounds derived from the fatty acid biosynthetic pathway in arthropods) to show the methyl branch pattern of each compound. Biosynthesis starts with a propionyl group (turquoise) rather than the common acetate group. This is evident from the location of the methyl group on even-numbered carbons. Elongation by malonate units extends the chain. Methyl branches are introduced by methylmalonate extender units. The box shows the common initial part of the biosynthesis. Deviations occur later, when a methylmalonate unit introduces the third methyl group in compounds 33A and 37A, but is lacking in 31A and 33B. Ether 37A adds two additional malonate units compared to 33A. A terminating methylmalonate unit introduces the third methyl group in 31A and 33B. Differentiation occurs by the additional chain extension by malonate in 33B compared to 31A. Biosynthesis is probably finished by reduction of an acid containing the complete carbon skeleton to the respective alcohol, followed by methylation to form the methyl ethers.

Figure 3 Behavioural isolation linked to chemotypes. Results for male silk extract choice trials. A, silk extract choice trial set-up (not drawn to scale). Males were released at the far end of the centre stick and given the choice between two female silk extracts, one on each cotton thread. B, map of Maui-Nui and Hawai'i showing where the spiders were collected for the choice trials and the chemotype of that population. All populations used in the choice trials were monotypic for a chemotype. C, results of male choice when given a choice between the silk extract of females from their own population (females depicted on the left of the table) or females from a different population either with the same or different chemotype (females depicted on top of the table). The degree of significance is indicated by asterisks above each plot (*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$).

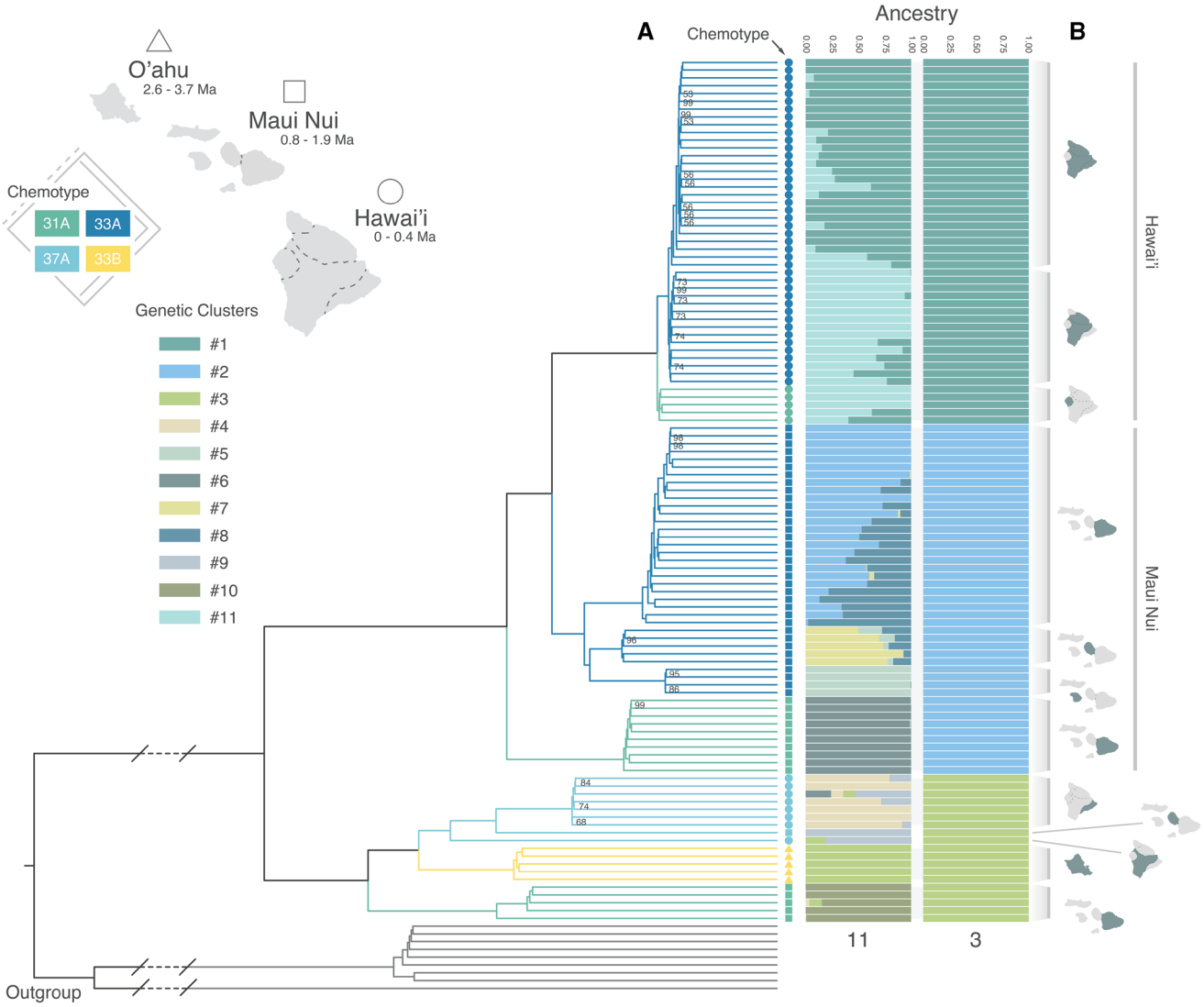

Figure 4 Phylogenetic relationship of the chemotypes. A, the phylogenetic tree reconstruction obtained by NgsDist. The chemotype of the individual is marked at the tips of the phylogeny with colour representing the specific chemotype and shape representing the island from where the spider was collected. The branches of the tree are also coloured according to the chemotype. Bootstrap values are provided for each node except for bootstrap values of 100 so as not to clutter the tree. B, population structure visualized using admixture plots for $K = 3$ and $K = 11$ (the best fit K). The admixture plot is aligned to the phylogenetic tree so that each individual's results align with that individual's position on the tree.

profile as themselves) (Fig. 3; Table S10). Furthermore, when the choice was between females with the same chemical profile but from two different locations, one being a female from the same location as the male and the other being from a different location, there was no significant difference in the frequency of choice between the two options (24 males out of the 50 tested chose the silk extract of the female from the same location as themselves) (Fig. 3; Table S10).

Individuals are genetically differentiated by chemotype

A total of 112 *T. quasimodo* spiders were successfully sequenced resulting in roughly 5.5 million SNPs that passed the quality filters and LD pruning (Table S11). A total of 194143191 SNPs were organically discovered, of which 162786753 were removed during the quality checks, leaving 31356438 filtered SNPs. Subsequent LD pruning removed 25844481 SNPs (82.42% of the filtered data) resulting in a final dataset of 5511957 SNPs, with an average sequencing depth of 12× and a range of 6–17×.

To assess the phylogenetic relationships among the different populations and chemotypes, a neighbour-joining tree was created (Fig. 4). Overall, the tree showed four major groups. The first group, which was the most derived group on the tree, consisted entirely of individuals from Hawai'i that had the 33A and 31A chemotypes. The individuals clustered by chemotype so that the 33A chemotype was sister to the 31A chemotype. The second group, which was sister to the first group, consisted of individuals from Lāna'i, West Maui, and East Maui. All individuals in this group had the 33A chemotype. In this group, the individuals all clustered by the specific island/volcano they were from with East Maui being the most derived followed by Lāna'i and West Maui, which

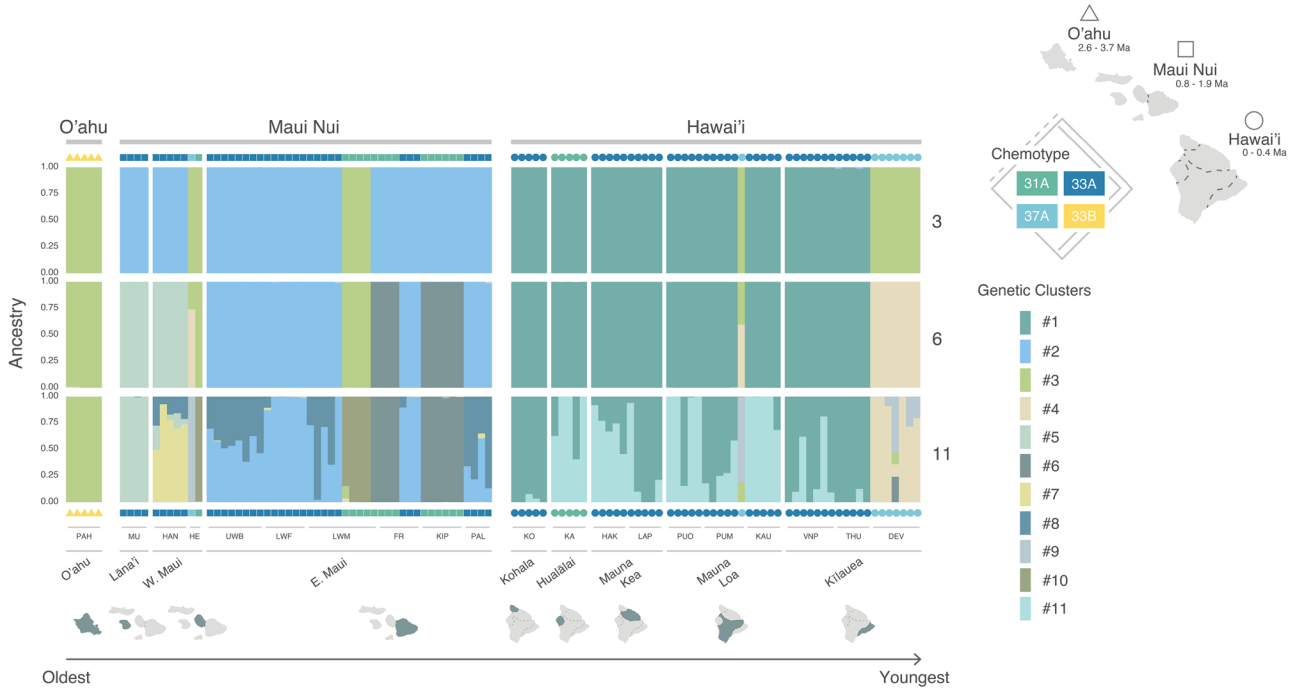

Figure 5 Population structure of the chemotypes. Population structure visualized using admixture plots for $K = 3$, 6, and 11. $K = 11$ was found to be the best fit K. The chemotype of the individual is marked at the top and bottom of the admixture plots with colour representing the specific chemotype and shape representing the island from where the spider was collected. The individuals are arranged in chronological order of the Hawaiian archipelago with the oldest island, O'ahu, on the far left to the youngest volcano, Kīlauea, on the youngest island, Hawai'i, on the far right. PAH, Pahole; MU, Munro; HAN, Hanaula; HE, Helu; UWB, Upper Waikamoi; LWF, Lower Waikamoi Flume; LWM, Lower Waikamoi Maile; FR, Flume Road; KIP, Kīpahulu; PAL, Palikū; KO, Kohala; KA, Kaloko; HAK, Hakalau; LAP, Laupāhoehoe; PUO, Pu'u O'o; PUM, Pu'u Maka'ala; KAU, Kaumana; VNP, Volcanoes National Park; THU, Thurston; DEV, Devastation.

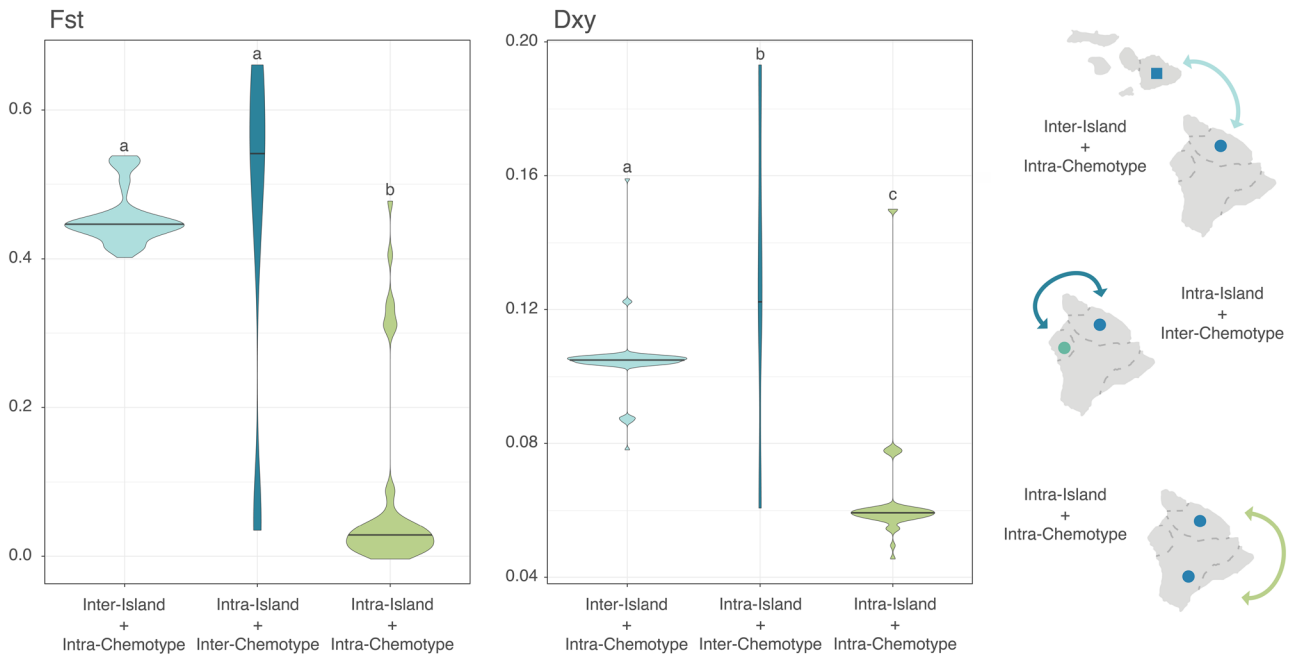

Figure 6 Genomic divergence by geography and chemotypes. Violin plots showing the F_{ST} and D_{xy} values for pairwise comparisons of all populations with more than one individual separated into three categories: inter-island/intra-chemotype, intra-island/inter-chemotype, and intra-island/intra-chemotype comparisons. The violin plots are coloured by these three comparison categories while the horizontal bars in the violin plots indicate the median and the letters above the plots indicate the results of the post-hoc Dunn's test.

were sister to each other. The third group, which was sister to the first two groups, consisted entirely of individuals from East Maui that had the 31A chemotype. Finally, the fourth group, which was sister to the rest of the groups, consisted of a mix of individuals from all three island groups (Hawai'i, Maui Nui, and O'ahu) as well as individuals with the 37A,

33B, and 31A chemotypes. All individuals in this group were also clustered by chemotype so that the 37A chemotype was sister to the 33B chemotype and the 31A chemotype was sister to the other two chemotypes.

Population structure analysis was conducted on $K = 2$–15 and revealed $K = 11$ to be the best supported number of distinct genetic clusters (Figs 5, S20). At $K = 3$, a number that represents the number of island groups in this study, two of the genetic clusters consisted entirely of individuals from a single island, either Hawai'i (cluster #1) or Maui Nui (cluster #2), while the third cluster consisted of a mix of individuals from all three island groups (cluster #3). All three clusters consisted of individuals of different chemotypes—33A and 31A for both cluster #1 and #2, and 37A, 33B, and 31A for cluster #3. Lastly, there was no shared ancestry among the three clusters. At $K = 6$, the fourth cluster formed out of the mixed island cluster (cluster #3) and consisted mostly of individuals from Hawai'i except for one individual from Maui Nui. The individuals in this cluster consisted entirely of the 37A chemotype. Two of the individuals from this fourth cluster shared ancestry with the third cluster. Additionally, two new clusters formed out of the Maui Nui cluster (cluster #2) and consisted of individuals from either Lāna'i and West Maui (cluster #5) or individuals from East Maui (cluster #6). Both clusters were monotypic for chemotype and consisted entirely of individuals of one chemotype—33A for cluster #5 and 31A for cluster #6. Neither cluster shared ancestry with the other clusters and vice versa. At $K = 11$, the best fit K, one of the new clusters (cluster #7) formed out of the previous Lāna'i and West Maui cluster (cluster #5) and largely separated the two islands. Furthermore, a new cluster (cluster #8) formed out of cluster #2 and created a more mixed genomic landscape in the spiders with the 33A chemotype across East Maui. Additionally, two new clusters (cluster #9 and #10) formed out of cluster #4 with cluster #9 consisting entirely of individuals with the 37A chemotype and cluster #10 consisting entirely of individuals with the 31A chemotype. In Hawai'i, a new cluster (cluster #11) formed out of cluster #1. Some individuals in cluster #1 and cluster #11, regardless of chemotype, shared ancestry with one another while some individuals did not. The PCA revealed similar results to the admixture plot and phylogenetic tree with the clusters on the PCA largely corresponding to that of the clusters in the admixture plot (Fig. S21). Further analysis using only samples from Hawai'i yielded very similar results of population structuring as the entire dataset (Figs S22, S23).

Overall, both geography (time in allopatry) and chemical signals were significantly correlated with genetic divergence. On Maui, chemical signals showed a stronger correlation with genetic divergence than geography, but this was not entirely the case on Hawai'i. D_{xy} and F_{ST} calculations revealed that genetic divergence across the intra-/inter-island/chemotype pairwise comparisons were significant for both relative (F_{ST}: Kruskal–Wallis $\chi^2 = 84.46$, d.f. = 2, $P < .001$) and absolute (D_{xy}: Kruskal–Wallis $\chi^2 = 84.39$, d.f. = 2, $P < .001$) genomic divergence (Figs 6, S24). Post-hoc Dunn's test with the Benjamini–Hochberg correction revealed that both inter-island/intra-chemotype and intra-island/inter-chemotype pairwise comparisons had significantly higher D_{xy} and F_{ST} than intra-island/intra-chemotype comparisons (Figs 6, S24; Table S12). Additionally, intra-island/inter-chemotype comparisons had higher D_{xy} and F_{ST} than inter-island/intra-chemotype comparisons but were not statistically significant ($P = .051$) (Figs 6, S24; Table S12). Genetic divergence across the intra-/inter-island/chemotype pairwise comparisons continued to be significant for both the relative and absolute divergence even when the pairwise comparisons were limited to those involving only Maui or Hawai'i populations (Maui F_{ST}: Kruskal–Wallis $\chi^2 = 47.49$, d.f. = 2, $P < .001$; Maui D_{xy}: Kruskal–Wallis $\chi^2 = 50.44$, d.f. = 2, $P < .001$; Hawai'i F_{ST}: Kruskal–Wallis $\chi^2 = 60.29$, d.f. = 2, $P < .001$; Hawai'i D_{xy}: Kruskal–Wallis $\chi^2 = 59.59$, d.f. = 2, $P < .001$) (Fig. S25; Table S13). In Maui, intra-island/inter-chemotype comparisons had significantly higher D_{xy} and F_{ST} than inter-island/intra-chemotype comparisons but this was not the case for Hawai'i (Fig. S25; Table S13).

Discussion

Our study found that there is substantial variation in the chemical profile of *T. quasimodo* spiders across populations, with males preferring their local chemical signal over different signals from other populations. Neither the variation in the chemical signal nor the mating preference was associated with genetic or geographical distance between populations, and chemical signals differed between close relatives within islands. Chemical signal differences were found even between taxa with minimal genetic divergence, suggesting their potential role in the initiation of reproductive isolation.

Assortative mate preference based on chemical signals

Marked genetic differentiation was observed between chemotypes of *T. quasimodo* both within and between islands and even within sites that were sympatric for multiple chemotypes. This is not surprising as male *T. quasimodo* showed a strong preference for the silk extract of females with the same chemotype. Strikingly, males did not differentiate between females from different islands nor from different volcanoes within an island when both females were of the same chemotype. This pattern suggests that mating preferences are mediated by chemical signals regardless of geographical and genetic distance. Neither signal divergence nor mating preference correlated linearly with genetic and geographical distance. This is further corroborated by the finding that individuals of a given chemotype on different islands were not closely related, but rather individuals of different chemotypes within an island were more closely related.

Chemical differences linked to mate recognition

The chemotype differences appear to be linked to mate recognition rather than the broad-scale abiotic environment. While the chemical profile in insects is frequently linked to physiological attributes given the dual function of cuticular

hydrocarbons in both desiccation tolerance and communication (Blomquist *et al.* 1987, Blomquist and Bagnères 2010, Gibbs and Rajpurohit 2010), this does not seem to be the case with the methyl ethers in these *Tetragnatha* spiders. There were no discernible trends in the climate in which the different *T. quasimodo* chemotypes were found nor were there any associations between the methyl ether compound structure and the climate that mirror those found in insect cuticular hydrocarbons (such as longer chain lengths and fewer methyl branches in warmer and drier climates) (Gibbs and Pomonis 1995, Gibbs *et al.* 1997, Rouault *et al.* 2004, Gefen *et al.* 2015, Krupp *et al.* 2020, Wang *et al.* 2022). A separate study looking at the methyl ethers found on the cuticle of Hawaiian *Tetragnatha* spiders from both the wet forest and the drier, hotter crater environment also hints at the possibility that the methyl ethers found in Hawaiian *Tetragnatha* spiders are not directly influenced by climatic variables such as temperature, humidity, and solar radiation (Adams 2022). While diet has been known to play a role in influencing the chemicals in insects (Geiselhardt *et al.* 2012, Kühbandner *et al.* 2012), this also does not seem to play a large role in the divergence of methyl ethers in *Tetragnatha* spiders. In the lab, spiders fed the same diet did not exhibit any changes in their methyl ether profiles (Adams *et al.* 2024). Further, gut content analysis of *Tetragnatha* spiders in Maui found that several species shared the same diet, yet these species, collected from the same location as the gut content study, were found to have vastly differing chemical profiles (Kennedy *et al.* 2019, Adams *et al.* 2024). Nevertheless, without further investigation at a fine-scale microhabitat level, it is not possible to rule out the role of cryptic niche differentiation in the differences between chemical profiles.

Chemical divergence precedes genetic divergence

As shown in previous work (Roderick *et al.* 2012), the genetic divergence of *T. quasimodo* implies isolation by both distance between the islands and island age, following the progression of colonization from older to younger islands, a phenomenon commonly seen in many island systems (Gillespie 2016, Shaw and Gillespie 2016). As expected, the *T. quasimodo* populations on the older islands show stronger genetic differentiation (average Maui intra-island $F_{ST} = 0.340$) while the populations on the youngest island, Hawai'i, show higher levels of genetic similarity and shared ancestry across populations (average Hawai'i intra-island $F_{ST} = 0.152$). Interestingly, this shared ancestry between populations on the youngest island is found even between populations with different chemotypes 33A and 31A (average pairwise $F_{ST} = 0.047$). This may indicate the importance of chemical signal divergence in the early, initiating stages of speciation where signal differences might accumulate prior to genetic differences. The early evolution of reproductive traits, especially sexual signalling traits, is well documented in a variety of taxa and there is increasing evidence that the evolution of sexual signals can outpace ecomorphological traits (Mendelson and Shaw 2005, Arnegard *et al.* 2010, Brand *et al.* 2020, Friis and Milá 2020, Turbek *et al.* 2021, Shaw *et al.* 2024). Further, a recent study investigating the genetic mechanisms of reproductive isolation in barn swallows found that the genes underlying the sexual signalling trait provided barriers to gene flow while gene flow across the remainder of the genome was less constrained (Schield *et al.* 2024). It is possible that the chemical signals in *T. quasimodo* behave similarly, playing a role in the initial divergence and reproductive isolation of populations.

Proposed biosynthesis pathway for signal divergence

Simple genetic architectures of sexual signals or the tight genetic linkage between signal and preference have been shown to facilitate the formation of sexually mediated isolating barriers in speciation (Ritchie and Butlin 2024). Consistent with many other *Tetragnatha* spiny leg species studied to date (Adams *et al.* 2024), the chemical profile of *T. quasimodo* spiders was simple—all four chemotypes were each made up of only one prominent methyl ether: either the 31A, 33A, 33B, or 37A methyl ether. The structures of these ethers were biosynthetically closely related. The proposed biosynthesis is explained in Figure 2. Common to all compounds is an initial part with methyl groups at the ω-9 and ω-15 positions. The letter ω denotes the alkyl end of the ethers, which is the biosynthetic starting point of compounds derived from the fatty acid biosynthetic pathway in arthropods (Holze *et al.* 2021). Ethers 33A and 37A share the third methyl group at ω-21 but differ in their chain length and the elongation of the number of carbons between the methoxy group and the nearest methyl branch. Ethers 31A and 33B introduce the third methyl group near the end of the chain but differ in their chain length. These structures suggest a close genetic similarity between the four chemotypes, with slight differences during the final stages of the biosynthetic chain construction. In insects, both the placement of methyl branches and the elongation are controlled by specific genes in the biosynthetic process—the microsomal fatty acid synthase genes and elongase genes, respectively (Holze *et al.* 2021). In *Drosophila simulans* and *D. sechellia*, changes in the expression of the elongase gene, *eloF*, were connected to changes in the length of cuticular hydrocarbons found on the surface of female flies, which in turn led to changes in the mating behaviour of male flies. The authors proposed that *cis*-regulatory differences in the two species may have led to the sexual isolation and speciation of *D. simulans* through changes in the chemical profile of the flies (Combs *et al.* 2018). In other cases, even the smallest change in the genes underlying chemical production has been shown to cause chemical signal divergence and behavioural isolation. In the European corn borer moth *Ostrinia nubilalis*, a fixed SNP in the pheromone production-controlling gene *pgfar* caused differences in the biosynthesis of the female pheromone blend, creating the two strains E and Z (Lassance *et al.* 2010). Furthermore, males are preferentially attracted to the pheromone blend produced by females of the same strain (Malausa *et al.* 2005, Coates *et al.* 2013). Similar relatively simple genetic changes in the

biosynthetic pathway of methyl ethers in *T. quasimodo* could explain the difference in the sexual signalling trait between populations.

Conclusion

The current study highlights the possible role of chemical signal divergence in the early, initiating stages of reproductive isolation in *T. quasimodo*. There was strong assortative mate preference among *T. quasimodo* based on the chemical signals, and the chemical differences were not linked to the abiotic environment, nor did they correlate with geographical distance. Chemicals differed between close relatives within islands and even between individuals with minimal genetic divergence and shared ancestry. Future work investigating chemical signal evolution of the entire Hawaiian *Tetragnatha* spiny leg lineage across the archipelago will allow us to examine the relative timing of chemical signal divergence in initiating and completing speciation and better understand the interplay between sexual signal divergence and ecological divergence.

In conclusion, adaptive radiations have been crucial in helping develop our understanding of speciation and how reproductive isolation may be attained and maintained between taxa. This study highlights the role of chemical signals in the reproductive isolation of an adaptive radiation of Hawaiian spiders. By investigating the relationship between the chemical signal divergence and genetic divergence of populations across a time gradient, we are a step closer to understanding how sexual signalling traits themselves initiate divergence in adaptive radiations.

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Author contributions

Seira Ashley Adams (Conceptualization [Equal], Data curation [Lead], Formal analysis [Lead], Funding acquisition [Equal], Investigation [Lead], Methodology [Equal], Project administration [Lead], Resources [Lead], Supervision [Lead], Validation [Lead], Visualization [Lead], Writing—original draft [Lead], Writing—review & editing [Equal]), Jennifer Lee (Investigation [Supporting], Methodology [Supporting], Resources [Supporting]), Freddy Gutierrez (Investigation [Supporting], Methodology [Supporting], Resources [Supporting]), Stefan Schulz (Formal analysis [Equal], Investigation [Equal], Methodology [Equal], Supervision [Supporting], Writing—review & editing [Equal]), Rosemary G. Gillespie (Conceptualization [Equal], Supervision [Equal], Writing—review & editing [Equal]), and Santiago R. Ramirez (Funding acquisition [Equal], Supervision [Equal], Writing—review & editing [Equal])

Supplementary material

Supplementary material is available at *Evolutionary Journal of the Linnean Society* online.

Conflicts of interest

The authors declare no competing interests.

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Data availability

Further information and requests for resources should be directed to and will be fulfilled by the corresponding author. All chemical extractions generated in this study were used for GC-MS analysis and therefore none is available for loan or use. Raw sequencing data are available on NCBI GenBank at PRJNA1445263. All data and code used have been deposited on Dryad and are publicly available at https://doi.org/10.5061/dryad.d51c5b0jm.

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