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Trophic Niche Differentiation within the Adaptive Radiation of Hawaiian *Tetragnatha* Spiders

By

Susan R Kennedy

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Abstract

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The Hawaiian *Tetragnatha* spiders are a remarkable and well-documented example of adaptive radiation. With *ca.* 50 species exhibiting a diverse range of morphologies and ecological affinities, all having evolved within the last few million years, this system has a great deal to offer for studies of evolutionary ecology. Furthermore, due to Hawaii's age-structured arrangement of islands, it is possible to infer past processes in this lineage's evolutionary history by using a space-for-time substitution. Although the ecological affinities of some Hawaiian *Tetragnatha*, notably the members of the Spiny Leg (actively hunting) clade, have been well documented, much is still unknown about the ecological niches of web-builders, and about the precise nature of ecological interactions that occur among both Spiny Leg and web-building species. Importantly, little is yet known about the dietary compositions of different Hawaiian *Tetragnatha* species. Diet is likely to play a critical role in the ecology of these spiders, all of which are obligate predators and therefore potentially compete for prey; and because the need to hunt prey can exert significant selective pressure, trophic ecology may also have played a part in driving the evolutionary diversification of this lineage.

The work presented here examines the trophic niche ecology of Hawaiian *Tetragnatha* in a geographic and evolutionary context. Working at multiple geographic scales – within a single forest site, among sites on an island, and across sites on three different islands – I characterize the trophic niches of several *Tetragnatha* species and probe the extent to which these niches overlap. I use multiple types of data to describe trophic niche, including web architecture (i.e., hunting behavior), stable isotope signatures of N and C in the spiders' tissues, and molecular identification of gut contents. Additionally, I present a phylogenetic hypothesis for the entire adaptive radiation, which lays the foundation necessary for analyzing ecological dynamics in the context of the spiders' evolutionary history. My work adds critical information to our knowledge of the natural history, evolutionary history and ecology of Hawaiian *Tetragnatha*, and builds on our understanding of the ecological and evolutionary dynamics of adaptive radiation.

This dissertation is dedicated to Dr. Leslie Bishop, who first opened the world of spiders to me, setting me on the path to complete this work. Leslie's brilliantly shining spirit and infectious love for all living things have shaped me into the person I am today.

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Introduction

The trophic niche is widely regarded as a key factor in the ecological segregation of species. While diet is only one axis of the ecological niche, it is a critical one in animals; and in many well-known examples of adaptive radiation, the phenomenon by which a lineage diversifies into multiple ecologically distinct species under selection (Schluter 2000), diet plays an essential role. The Galápagos finches occupy a broad range of ecological niches based on their bill size and shape, which are associated with their diet (Grant and Grant 2011); Hawaiian honeycreepers show a similar pattern of dietary specialization relating to bill morphology (Lovette *et al*. 2002); African cichlid fishes show trophic specialization that correlates with interspecific differences in pharyngeal jaw morphology (Muschick *et al*. 2012). In all of these systems, diet appears to be an important axis of divergence among closely related species.

On the Hawaiian Islands, there exists another particularly rich and promising system for the study of trophic niche divergence in an evolutionary context: the *Tetragnatha* (long-jawed orbweaver) spiders. This remarkable example of adaptive radiation consists of an estimated 50 or more species (Gillespie 2015), which display an impressive variety of morphological, ecological and behavioral phenotypes not found elsewhere in the range of this globally distributed genus. Two major clades, each associated with a different hunting strategy, are known from Hawaii: the web-builders (Simon 1900, Gillespie 1992, 1994, 2003), which construct two-dimensional orb webs for prey capture, and the Spiny Leg clade (Gillespie 1991, 2002), which do not spin webs and instead hunt actively. Within each of these groups, particularly the Spiny Leg clade, researchers have found striking examples of ecological divergence among close relatives, and ecological and morphological convergence in multiple lineages across the islands (Gillespie 2004, Blackledge and Gillespie 2004). What makes this system especially suited for studying the ecological dynamics of adaptive radiation is the ability to incorporate the temporal axis: because the Hawaiian Islands are arranged along a substrate age gradient due to their volcanic origin (Carson and Clague 1995), it is possible to use patterns and processes occurring on younger islands to infer the history of older islands. Using this age structure, we can see how niche ecology changes over evolutionary time, and how evolutionary and ecological processes have each contributed to the diversification of lineages across the Hawaiian Archipelago.

This dissertation focuses on the ecology of Hawaiian *Tetragnatha* spiders, with a particular emphasis on diet, to examine the ways in which ecological and evolutionary processes have interacted in this system to produce the remarkable diversity of Hawaiian *Tetragnatha* we see today. I use multiple types of ecological data – hunting behavior, microhabitat, chemical signatures, and gut content analysis – in a phylogenetic context to attempt to understand how trophic ecology can drive evolution, and vice versa, in this system.

In **Chapter 1**, I begin to explore the ecology of Hawaiian *Tetragnatha* in an age-structured geographic context. Using three sites of different substrate age on the Big Island, ranging from *ca.* 200 to 20,000 years, I test the extent to which chemical signatures of substrate are borne up through higher trophic levels: specifically, *Tetragnatha* spiders and another spider genus, *Ariamnes*, which specializes on eating other spiders. To determine how closely these predatory animals reflect the stoichiometry of the sites from which they were collected, I measure the stable nitrogen and carbon isotope ratios of spiders' tissues. I find a remarkable pattern of increasing δ^{15}N – the ratio of heavier to lighter stable N isotope – with substrate age in both *Tetragnatha* and *Ariamnes* spiders. The changes in δ^{15}N of spiders along this age gradient appear to correspond to biologically available N pools at each of these sites. The strong chemical signature of substrate suggests a high level of isolation among spider populations, even when their habitats are in close geographic proximity. Such isolation is expected to have played an important role in the evolutionary diversification of Hawaiian *Tetragnatha* as well as *Ariamnes* spiders. Additionally, I find a consistent pattern of higher δ^{15}N in web-building than in Spiny Leg *Tetragnatha* at all sites, suggesting a fundamental difference in diet, metabolism, silk production and recycling, or some other factor between these two major clades of Hawaiian *Tetragnatha*.

In **Chapter 2**, I zoom in on a community of *Tetragnatha* spiders in East Maui to examine their trophic niches in relation to one another, using four different types of data to characterize the niche: web structure, web placement (microhabitat), stable isotopes of N and C, and gut content metabarcoding. I test for trophic niche partitioning among five web-building *Tetragnatha* species, as well as broad niche differences between web-building and Spiny Leg spiders in the habitat. Results of this study strongly suggest trophic niche partitioning among sympatric web-building *Tetragnatha* species, based on distinctive interspecific differences in web architecture and placement and stable isotope signatures. I also find differences in gut content between web-building and Spiny Leg species, and – matching the pattern I found in Chapter 1 – a higher δ^{15}N in web-building than in Spiny Leg spiders. My findings support the hypothesis that ecological niche partitioning is occurring among the species within this diverse community of *Tetragnatha* spiders.

Chapter 3 provides an evolutionary context for this work by presenting a phylogenetic hypothesis for the Hawaiian *Tetragnatha* radiation. This is the most taxonomically comprehensive phylogeny of this radiation to date. I use ten molecular markers totaling approximately 3300 base pairs of DNA, including both nuclear and mitochondrial loci, amplified with PCR and sequenced on an Illumina MiSeq platform. Using the resultant Maximum Likelihood and Bayesian trees, I explore patterns of ecological and morphological divergence within islands; convergent evolution of phenotypes in multiple lineages and on multiple islands; and ecological niche conservatism in some clades. In addition, I revisit the long-standing question of whether the web-building *Tetragnatha* form a clade with the Spiny Leg *Tetragnatha*, and find evidence that the Hawaiian *Tetragnatha* adaptive radiation likely originated from multiple colonization events. My results indicate that numerous different processes interacted in complex ways over the evolutionary history of Hawaiian *Tetragnatha*, producing contrasting patterns of divergence, convergence and niche conservatism in different lineages within the radiation.

Finally, **Chapter 4** examines trophic niche relationships in Hawaiian *Tetragnatha* from three different islands, using the age gradient of the islands to test for temporal changes in niche dynamics over evolutionary time. I use a novel method of molecular gut content analysis to identify the taxonomic composition of prey of 23 different *Tetragnatha* species collected from the Big Island of Hawaii, Maui, and Oahu. According to theory (Schluter 2000), adaptive radiation should be characterized by a decrease over time in the niche breadths of species, which are driven toward ecological specialization as species accumulate and compete for resources. Because of Hawaii's geologic age structure, it is possible to test this hypothesis by looking at communities in very young sites (Big Island) versus intermediate-aged (Maui) and older sites (Oahu), and by assessing patterns of niche ecology along this age gradient. Although I do not find any significant decrease in niche breadth over time, I do uncover intriguing patterns of trophic niche interaction changing with island age: while younger spider communities appear to have high dietary overlap, species in older communities show evidence for niche partitioning. My results support the fundamental importance of diet as an axis of niche divergence, as well as upholding the expectation that co-occurring species in adaptive radiations become more ecologically divergent from one another over time.

Together, these four chapters provide a detailed view of the trophic ecology of Hawaiian *Tetragnatha* spiders, and suggest some ways in which the interplay of ecological and evolutionary processes may have contributed to these spiders' current diversity. While much remains to be discovered about this remarkable system, my work builds on our knowledge of these spiders' natural histories and interactions with one another, and contributes to our growing understanding of the processes that drive evolutionary diversification.

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Chapter 1

Stable isotopes of Hawaiian spiders reflect substrate properties along a chronosequence

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Abstract

The Hawaiian Islands offer a unique opportunity to test how changes in the properties of an isolated ecosystem are propagated through the organisms that occur within that ecosystem. The age-structured arrangement of volcanic-derived substrates follows a regular progression over space and, by inference, time. We test how well documented successional changes in soil chemistry and associated vegetation are reflected in organisms at higher trophic levels – specifically, predatory arthropods (spiders) – across a range of functional groups. We focus on three separate spider lineages: one that builds capture webs, one that hunts actively, and one that specializes on eating other spiders. We analyze spiders from three sites across the Hawaiian chronosequence with substrate ages ranging from 200 to 20,000 years. To measure the extent to which chemical signatures of terrestrial substrates are propagated through higher trophic levels, we use standard stable isotope analyses of nitrogen and carbon, with plant leaves included as a baseline. The target taxa show the expected shift in isotope ratios of δ^{15}N with trophic level, from plants to cursorial spiders to web-builders to spider eaters. Remarkably, organisms at all trophic levels also precisely reflect the successional changes in the soil stoichiometry of the island chronosequence, demonstrating how the biogeochemistry of the entire food web is determined by ecosystem succession of the substrates on which the organisms have evolved.

Introduction

Evolutionary processes are determined in large part by the ecosystems within which they take place. While connecting the processes of evolutionary biology and ecology remains a critical frontier in biological sciences (Matthews *et al*. 2011), there are few studies that demonstrate how mechanisms driving processes of evolutionary biology and ecosystem science are linked. The current study seeks to understand how organismal diversity may reflect successional shifts in soil chemistry by testing the extent to which organisms at different trophic levels reflect the properties of the substrates on which they occur.

The Hawaiian Archipelago presents a highly suitable system for studying the link between evolutionary processes and ecosystem properties. The current high islands of Hawaii are arranged sequentially from oldest to youngest, with Kauai, at 5.1 million years, in the far northwest, and Hawaii Island, at <1 million years, in the southeast (Carson and Clague 1995). This sequential order is a consequence of the archipelago being located on a volcanic hot spot, where magma upwelling from the earth's mantle has formed into large shield volcanoes. At the same time, the tectonic plate on which the islands are situated is moving toward the northwest such that each newly emerging island has appeared to the southeast of its next-oldest neighbor. The resultant, nearly-linear age gradient makes Hawaii an ideal chronosequence: a temporally varied system in which the ecosystems of the younger sites are currently developing in a manner assumed to reflect the developmental history of the older sites (Walker *et al*. 2010). Given fairly precise information on the age of formation and subsequent history across a chronosequence, these systems can provide unprecedented insights into ecosystem development. Thus, chronosequences have added significantly to our understanding of how nutrients change over time (Vitousek 2004) and the impact of changes in soil nutrient availability on plants (Wardle *et al*. 2008), decomposers (Williamson *et al.* 2005, Doblas-Miranda *et al*. 2008), above-ground and below-ground ecosystem processes (Wardle *et al.* 2004), and entire arthropod communities (Gruner 2007).

Hawaii has served as a chronosequence for detailed studies on the ways in which ecosystem properties and functions change over extended time (Vitousek 2004). Nutrient flow and its impacts on primary producers (trees) have been well characterized in this system. Studies have examined the evolution of soils on substrates of different surficial age (300 y – 4.1 Mya), controlled for elevation, climate, land use history, and canopy vegetation (*Metrosideros polymorpha*), with all minerals derived from volcanic ash. An important finding of this work was that soil nitrogen, foliar nutrient availability and productivity start off very low, increase rapidly with substrate age, peak on substrates of intermediate age (*ca*. 20,000 y) on the youngest island, and then decline rapidly on older islands before all but disappearing on the oldest (Vitousek *et al.* 1989, 1995, 1997). A more recent study found that tree height and canopy nitrogen also peak on intermediate-aged (20,000 y) substrates on the youngest island (Vitousek *et al*. 2009). Nitrogen isotopes follow a similar pattern, with foliar δ^{15}N very low at the youngest sites, increasing with substrate age, and highest at a 67,000 y site (Vitousek *et al.* 1989).

Geologic history and nutrient flow evidently have important effects on the lowest trophic level – plants – but little is known about how these effects might be propagated through higher trophic levels, i.e., higher-level consumers. At the same time, work on the effects of substrate age on above-ground systems, including whole communities, has shown that community traits such as population species diversity (Gillespie and Baldwin 2010, Lim and Marshall 2017), genetic structure (Roderick *et al*. 2012), and network modularity (Rominger *et al*. 2016) change in a predictable manner across the Hawaiian chronosequence. However, although these community-level studies have markedly enhanced our understanding of the changes in community ecology over time, there has as yet been no attempt to link chemical changes during the evolution and development of soils (and associated ecosystem properties) with the abundance, diversity, and evolutionary histories of above-ground organisms. The current study begins to address this question by testing the effects of substrate age on the biochemistry – isotopic signatures – of secondary consumers (predators) across a Hawaiian chronosequence.

The application of stable isotope information has revolutionized studies of nutrient flow and niche ecology in a wide range of organisms (e.g. Fry 1988, Hobson and Welch 1992, Muschick *et al.* 2012). In particular, nitrogen and carbon stable isotopes have been found to reflect trophic position: both δ^{15}N and δ^{13}C tend to increase in a predictable manner with each successive trophic level (Post 2002, but see deVries *et al*. 2015). Stable isotopes have also been used to track nutrient flow, climatic shifts, and migration patterns in a variety of ecological systems (Best and Schell 1996, Chamberlain *et al*. 1997, Iacumin *et al.* 2005, McMahon *et al*. 2016). The current study uses stable isotopes of N and C to assess the extent to which entire food webs are influenced by the chemistry of their habitats. We chose to focus on spiders because they are mobile generalist predators and encompass multiple trophic levels, from feeding on herbivorous insects to specializing on the highest predator levels among Hawaiian arthropods (other spiders). This substantial variation in trophic ecology allows us to test how functional and trophic differences are reflected in isotopic signatures, and the extent to which the biogeochemistry of a food web is determined by the chemistry of the immediate substrate. The use of N and C stable isotopes is especially well suited to this study because of the predictable manner in which both elements can reflect trophic position, and because of the importance of nitrogen in ecosystem development (Boring *et al*. 1988).

We analyzed Hawaiian spiders belonging to two lineages within the adaptive radiation of long-jawed orb-weavers (*Tetragnatha*, Tetragnathidae) and one lineage within the stick spiders (*Ariamnes*, Theridiidae). The *Tetragnatha* radiation includes ca. 60 species, which display a spectacular array of colors, shapes, sizes, behaviors, and ecological affinities not observed elsewhere in the range of this genus (Blackledge and Gillespie 2004, Gillespie 2004, 2015). The radiation consists of two major clades: one that spins webs for prey capture ("web builders"), and another that has lost the web-spinning behavior and instead hunts actively (the "Spiny Leg" clade; Gillespie 1991, 2002b). Observational data indicate that both web-building and Spiny Leg *Tetragnatha* feed on a mixture of insect herbivores and predators (Binford 2001), although the exact composition of these spiders' diets has not yet been fully characterized. The Hawaiian *Ariamnes*, currently represented by 11 known species across the Hawaiian Islands (Gillespie and Rivera 2007), are also ecologically diverse and largely araneophagic (i.e., preying on other spiders) (Gillespie *et al*. 2018). Like *Tetragnatha*, these spiders are exclusively nocturnal, and like the Spiny Leg *Tetragnatha*, they hunt without the use of a web.

Given the contrasting hunting strategies (web-building versus active hunting) and trophic positions (generalist versus araneophagic) across these spider lineages, the three groups vary predictably in their position in the food web, from largely feeding on primary consumers (i.e., insect herbivores) to exclusively feeding on secondary and higher consumers (i.e., spiders). Within this system, we tested the hypothesis that isotopic signatures of spiders should reflect the biogeochemistry of their respective habitats, from young to older in the Hawaiian chronosequence. Thus, not only should the different spider lineages illustrate now-standard expectations for isotope signatures associated with rising trophic levels, but the trophic ecology of the entire food web should reflect changes in soil chemistry across the chronosequence. In particular, given that δ^{15}N in the soil increases during the building phase of the Hawaiian ecosystems (Vitousek *et al*. 1997), we expect δ^{15}N to be lowest in the spiders at the youngest (200-750 y) site and highest in the spiders at the oldest (20,000 y) site.

Methods

Study sites

Hawaii's sequential age structure has made it an ideal system for previous studies on soil evolution and nutrient cycling, wherein the Long Substrate Age Gradient (LSAG) was established (Crews *et al*. 1995, Vitousek 2004). This study focuses on Hawaii Island, the youngest in the archipelago, because the largest possible range of N availabilities is expected to be found there: soil nitrogen is lowest in the youngest substrates and peaks in the older substrates on Hawaii Island before declining on the older islands (Vitousek *et al.* 1995).

Specimens were collected on the windward side of Hawaii Island under permits from the State of Hawaii Department of Land and Natural Resources (endorsement # FHM14-349) and the National Park Service (study # HAVO-00425). Three sites of different substrate age, chosen for their comparable elevations and climates as well as the overlap of two of the sites ('Ola'a and Laupāhoehoe) with those characterized based on soils (Vitousek *et al*. 1997), were sampled (see Fig. 1). Substrate ages were determined based on data from the United States Geological Survey (Sherrod *et al*. 2007) and from the LSAG (Crews *et al*. 1995, Vitousek 2004). All three sites are wet/mesic forest dominated by *Metrosideros polymorpha* and *Acacia koa* in the canopy, with *Cibotium* spp. dominating the understory. The sites range from 1,180 to 1,390 m in elevation, with mean annual temperatures of 13.9 to 15.4 degrees Celsius and mean annual rainfall of 3,035 to 3,090 mm (Giambelluca *et al*. 2014).

Upper Waiakea is a very young site on a 200- to 750-year-old lava flow in a stratified matrix of differently-aged substrates within the Upper Waiakea Forest Reserve, off of Stainback Highway on Mauna Loa. 'Ola'a Forest is on an older lava flow on Kilauea, situated within Hawaii Volcanoes National Park. The trees in 'Ola'a are rooted in a thick layer of tephra of approximately 2,100 years old (Vitousek 2004), beneath which is an older flow of 5,000-11,000 y (see Fig. 1). Although the United States Geological Survey (USGS) classifies this substrate as 5,000-11,000 y, we consider its biota to be influenced by the chemical properties of the 2,100-year-old tephra in which the forest is rooted. 'Ola'a is therefore approximately one order of magnitude older than Upper Waiakea, yet is located just 11.5 km S of the younger site. This proximity makes the two sites especially useful for measuring effects of habitat age on a small geographical scale. The oldest site in this study is Laupāhoehoe, located in the Laupāhoehoe Experimental Forest Unit on Mauna Kea. While USGS data (Sherrod *et al*. 2007) estimate the lava flow age at 5,000-11,000 y, the sampling locality overlaps with an LSAG site which has been studied in great detail and whose forest is rooted in a layer of soil believed to be approximately 20,000 y old (Vitousek 2004, Vitousek *et al*. 2009). We therefore follow the LSAG classification of 20,000 y for Laupāhoehoe.

Collections at each site were centered at the following coordinates, with searches extending up to 100 m in any direction:

- Upper Waiakea: 19.562°N, 155.272°W
- 'Ola'a: 19.462°N, 155.248°W
- Laupāhoehoe: 19.922°N, 155.301°W

Specimen collection

Six species of *Tetragnatha* spiders (*Tetragnatha anuenue*, *T. brevignatha*, *T. hawaiensis*, *T. perkinsi*, *T. quasimodo* and the undescribed species *T.* sp. “Golden Dome”) and two species of

Ariamnes spiders (*Ariamnes hiwa* and *A. waikula*) were collected in the field from 11 March to 18 April 2015, and from 6 to 15 February 2016. All study species are morphologically distinct and can be easily identified in the field. Plant samples were leaves of dominant or common forest vegetation (*Metrosideros polymorpha*, *Cibotium* spp. and *Cheirodendron* spp.), all of which are also easily identified on sight. After a preliminary analysis, it was determined that leaf litter should be added to the study in order to help explain differences in carbon isotope values. Unfortunately, permitting and time constraints only allowed for leaf litter to be collected from the youngest site (Upper Waiakea) on 15 October 2016.

Spiders were individually hand-captured into clean plastic snap-cap vials which were labeled on the outside with unique identifiers, while plant leaves were clipped off with scissors and stored in labeled paper envelopes. In Upper Waiakea, leaf litter was also collected from the bases of *M. polymorpha* trees and placed in paper envelopes. Leaves were air-dried in their envelopes in a sealed container of silica for three weeks prior to processing. Leaf litter was dried in a 60° C oven overnight.

Spiders were photographed up close using a Nikon D5200 with an AF-S DX Micro-NIKKOR 40 mm f/2.8 g lens and a Speedlight SB-400 flash from both dorsal and lateral angles. This created a photographic voucher and allowed for visual identification of species, sex, and maturity. Spiders were killed overnight in a freezer and air-dried in separate snap cap vials, each with one clean bead of silica gel, before being transferred to individual 2-mL centrifuge tubes.

Stable isotope analysis

Individual dried spiders were weighed into 9 x 5 mm pressed tin capsules for isotopic analysis. To optimize N content for isotopic analysis, a 1.5-mg mass was recommended for each sample (S Mambelli, UC Berkeley Center for Stable Isotope Biogeochemistry, pers. comm., 2014). Due to the spiders' small body size (ranging from ca. 0.4 to 5 mg dry weight for the majority of specimens), it was not feasible to obtain sufficient material from individual body parts, although it has been found that different spider tissues can undergo different isotopic turnover rates (Belivanov and Hambäck 2015). Therefore, in order to control for possible variation in isotopic turnover among tissue types, the spiders' entire bodies were analyzed. When spiders exceeded 2.5 mg dry weight, they were homogenized (powdered and mixed) with a mortar and pestle, and a 1.5-mg sample of the homogenized tissue was used. For smaller spiders (<2.5 mg), the whole intact body was packed into the tin capsule in order to avoid excessive loss of material. Plant leaves were individually homogenized in a Mini-Beadbeater (BioSpec Model 8) in 7-mL tubes with stainless steel ball bearings, then weighed into 9 x 5 mm tin capsules. Due to the relatively low N:C ratio of plants, 6 mg of material was weighed out for each leaf sample. Leaf litter was processed in the same manner as plant leaves.

Samples were analyzed for nitrogen and carbon content (% dry weight) and nitrogen and carbon stable isotope ratios via elemental analyzer/continuous flow isotope ratio mass spectrometry using a CHNOS Elemental Analyzer (model: Vario ISOTOPE cube; Elementar, Hanau, Germany) coupled with an IsoPrime 100 mass spectrometer (Isoprime Ltd, Cheadle, UK). The isotope ratio is expressed in “delta” notation (in parts per thousand, or ‰ units). The isotopic composition of a material relative to that of a standard on a per mill deviation basis is given by $\delta^{15}N$ (or $\delta^{13}C$) = (R_{sample} / $R_{standard}$ – 1) x 1,000, where R is the molecular ratio of heavy to light isotopes. The standard for nitrogen is air. The standard for carbon is V-PDB. The reference material NIST SMR 1547 (peach leaves) was used as calibration standard (long-term precision (since 2000) using this standard is ±0.07‰ for both N and C isotope analyses). All isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. Long-term external precision based on reference material NIST SMR 1577b (bovine liver) is 0.15‰ and 0.10‰, respectively, for N and C isotope analyses.

Data analysis

Results from the isotopic analysis were categorized into the following functional groups: “plant” (foliar samples of the genera *Metrosideros*, *Cibotium* and *Cheirodendron*); “Spiny Leg” (Spiny Leg *Tetragnatha* species: *T. anuenue*, *T. brevignatha* and *T. quasimodo*); “web” (*Tetragnatha hawaiensis*, *T. perkinsi*, and the undescribed species nicknamed *T.* “Golden Dome”); and “*Ariamnes*” (*Ariamnes hiwa* and *A. waikula*, the two species found on Hawaii Island (Gillespie and Rivera 2007)). Although all spider species were initially analyzed separately, the results showed that the data grouped taxa together, largely in accordance with functional group. Because members of one group (e.g., web-builders) were closer to one another than to other groups (e.g., Spiny Leg), we chose to focus on these broader ecological categories – functional groups – rather than species.

Effects of site and functional group on $\delta^{15}N$ and $\delta^{13}C$ were tested using a 2-way Anova allowing for interaction (with site and functional group as factors) on R statistical software (version 3.2.2, 64-bit). Main effects were then analyzed separately: we tested (1) effect of site within each functional group and (2) effect of functional group within each site. When significant differences were found, pairwise comparisons were made using Tukey's honest significant difference test (Tukey 1949).

Results

We found a significant interaction between site and functional group for $\delta^{15}N$ (F = 8.615, $p < 0.001$), but not for $\delta^{13}C$ (F = 1.841, p = 0.092; Table 1). Significant main effects were found for all variables tested: site for $\delta^{15}N$ (F = 692.1, $p < 0.001$), functional group for $\delta^{15}N$ (F = 113.6, $p < 0.001$), site for $\delta^{13}C$ (F = 55.51, $p < 0.001$), and functional group for $\delta^{13}C$ (F = 95.15, $p < 0.001$).

Main effects: site

For $\delta^{15}N$, a significant site effect was found within all functional groups (plants: F = 78.74, $p < 0.001$; Spiny Leg: F = 446.9, $p < 0.001$; web-builders: F = 216.6, $p < 0.001$; *Ariamnes*: F = 80.87, $p < 0.001$; Table 2 and Fig. 2). We performed a Tukey's HSD test to reveal pairwise differences among sites. $\delta^{15}N$ showed a clear pattern of stepwise increase with substrate age (lowest in Upper Waiakea (200-750 y), intermediate in 'Ola'a (2,100 y), and highest in Laupāhoehoe (20,000 y)). This pattern held true for every functional group; the only comparison not found to be significant was 'Ola'a vs. Laupāhoehoe within *Ariamnes* (Tukey's adjusted $p = 0.203$).

For $\delta^{13}C$, a significant site effect was found within every functional group except for plants (plants: $F = 0.7997$, $p = 0.482$; Spiny Leg: $F = 5.681$, $p = 0.005$; web-builders: $F = 31.91$, $p < 0.001$; *Ariamnes*: $F = 36.62$, $p < 0.001$; Table 2 and Fig. 3). Among the three groups of spiders, $\delta^{13}C$ was significantly lower in Laupāhoehoe (20,000 y) than in 'Ola'a (2,100 y).

Main effects: functional group

A significant functional group effect was found in all sites for $\delta^{15}N$ (Upper Waiakea: $F = 68.38$, $p < 0.001$; 'Ola'a: $F = 34.23$, $p < 0.001$; Laupāhoehoe: $F = 28.90$, $p < 0.001$; Table 3 and Fig. 2). A Tukey's HSD test found significant differences among every pair of groups except for the following pairs in Upper Waiakea: web-builders vs. *Ariamnes* (Tukey's adjusted p-value = 0.071), web-builders vs. leaf litter (Tukey's adjusted p-value = 0.998), and *Ariamnes* vs. leaf litter (Tukey's adjusted p-value = 0.512).

For $\delta^{13}C$, a significant functional group effect was found at all three sites (Upper Waiakea: $F = 36.42$, $p < 0.001$; 'Ola'a: $F = 41.29$, $p < 0.001$; Laupāhoehoe: $F = 41.48$, $p < 0.001$; Table 3 and Fig. 3). At all sites, plants were significantly lower in $\delta^{13}C$ than the next-lowest trophic level, Spiny Leg spiders (consumers). In 'Ola'a (2,100 y), Spiny Leg spiders had significantly lower $\delta^{13}C$ than either web-builders or *Ariamnes* (Tukey's adjusted p-value < 0.001). In Upper Waiakea (200-750 y), although there was no significant difference between *Ariamnes* and either of the *Tetragnatha* functional groups, the Spiny Leg *Tetragnatha* were significantly lower in $\delta^{13}C$ than web-builders (Tukey's adjusted p-value < 0.001). In Laupāhoehoe (20,000 y), no significant difference was found between any pair of spider groups.

Discussion

The results from this study provide a novel perspective on how changes in the substrate chemistry of the terrestrial land surfaces across a chronosequence of developing ecosystems are propagated up through an entire food web. To our knowledge, this is also the first study to characterize the isotopic signatures of different ecological groups represented by exemplary adaptive radiations of spiders in Hawaii.

Parallel shifts in isotopic signature across the chronosequence of ecosystem development

Previous work on the ecological characteristics of forests across the chronosequence has detailed the evolution of Hawaiian ecosystems in the context of soil properties and vegetation (Vitousek *et al.* 1989, 1995, 1997). Our results show that the chemical signatures of nutrient availability that characterize a given site are borne all the way up to the highest trophic levels – top predators

– on a Hawaiian chronosequence. Indeed, the δ^{15}N values of spiders perfectly match expectations for their respective habitats. Where nitrogen is most limited – at the youngest site (Upper Waiakea) – spiders have the lowest values of δ^{15}N; as substrate age and nitrogen availability increase, so too does the δ^{15}N of spiders. This makes sense physiologically because when nitrogen is very limited, the lighter isotope (^{14}N) is not as easily lost in reactions, and is instead retained at a greater rate in an organism's tissues (Austin and Vitousek 1998). Conversely, when biologically available nitrogen is very abundant, ^{14}N is readily lost (e.g., in excretion), leaving behind a greater proportion of the heavier ^{15}N in the organism's tissues. Thus, our results support the hypothesis that the isotopic signatures of the spiders – as well as the plants – track the changes in the geological age of the islands (Sherrod *et al*. 2007) and the associated changes in nitrogen in soils across the Hawaiian chronosequence measured by Vitousek *et al*. (1997).

While it might not be surprising that the increase in δ^{15}N in the spiders tracks the increases in plants across the geological gradient, the fact that the relationship is so tight is remarkable, as it suggests that even higher-level consumers (predators) reflect the δ^{15}N of the immediate site. This result is especially notable because it was found in cursorial animals, which, because of their mobility, might be expected to show only a weak association with the substrates on which they were collected. Instead, the spiders carry clear signatures of their immediate ecosystem. Given that the sites that were sampled are in very close proximity (11.5 km between 'Ola'a and Upper Waiakea), and not separated by any significant physical barrier, the results suggest an extraordinary level of isolation among spider populations. This has implications for the mechanisms by which the *Tetragnatha* adaptive radiation may have arisen: Isolation between populations separated by short distances can serve as a crucible for evolution (Carson *et al.* 1990). Perhaps the same mechanisms that are currently at work on Hawaii's youngest island also led to the rise of the approximately 60 endemic *Tetragnatha* species found across the archipelago today. Results for *Ariamnes* were similar, all higher than the other spider lineages, and values increasing with substrate age (though the increase from 'Ola'a to Laupāhoehoe was not significant, presumably due to a relatively small sample size (n = 9) at these sites). *Ariamnes*, like the *Tetragnatha*, has undergone a substantial, though smaller at 11-16 species, adaptive radiation across the islands (Gillespie *et al*. 2018).

The carbon isotope data show a less clear pattern than nitrogen, but nevertheless indicate site-specific differences among the spiders. Notably, the plant samples did not differ significantly in δ^{13}C among the three sites, suggesting that perhaps δ^{13}C does not accurately reflect nutrient differences among the substrates. By contrast, δ^{15}N appears to strongly reflect nutrient availability at the different sites. However, spiders did consistently show higher δ^{13}C in 'Ola'a (2,100 y) than in Laupāhoehoe (20,000 y). Thus, the relationship between baseline (plant) signatures and higher predator (spider) signatures is weaker in carbon than in nitrogen. This suggests that spiders at the three sites may not be consuming exactly the same assemblages of prey, perhaps due to variations in the availability of different insect (potential prey) species at different sites. A detailed study of the precise compositions of these spiders' diets, using either molecular gut content analysis (e.g., Krehenwinkel *et al*. 2017) or an isotopic mixing model with robust sampling of the entire arthropod community, could greatly enhance our understanding of the processes that account for the differences in δ^{13}C among spider populations.

Trophic positions

Our stable nitrogen isotope data reflect the different functional roles and trophic positions of the Hawaiian spiders. Our results are consistent with the enrichment of the heavier isotope, ^{15}N, at higher trophic levels, with plants having the lowest values of δ^{15}N, *Tetragnatha* having intermediate values, and the spider-eating *Ariamnes* having the highest. Additionally, we found that the δ^{15}N of the Spiny Leg (cursorial) spiders was consistently lower than that of web-building *Tetragnatha*. A simple explanation for the difference in δ^{15}N in cursorial vs. web-building spiders may be that the different functional groups consume different prey. Cursorial spiders are likely to interact with abundant insect herbivores, while web-builders may trap a larger proportion of flying insects at higher trophic levels, such as hymenopteran or dipteran parasitoids, decomposers, or predators. This dietary difference has been used to explain the phenomenon of a higher δ^{15}N in web-builders compared to cursorial spiders in a forest hedge community (Sanders *et al.* 2015). Another possible explanation is that the difference is due to the manufacturing of the orb web itself. Given that webs are an excretory product, and that excretion tends to favor the lighter ^{14}N, it may be that the higher levels of "excretion" lead to an enrichment in ^{15}N in the bodies of web-builders compared with cursorial spiders. A number of previous studies suggest such an effect. For example, across a community of web-building riparian spiders, lower δ^{15}N was found in *Miagrammopes* (Uloboridae; Kelly *et al.* 2015), a genus characterized by a reduced capture web (often just a single line; Lubin *et al.* 1978), than in other spiders. Likewise, a study of niche width across a guild of spiders showed that cursorial spiders consistently had the lowest δ^{15}N, while orb web spiders had the highest (Sanders *et al.* 2015). In each of these systems, the cursorial spiders showed the highest levels of intraguild predation (i.e., feeding on other spiders), indicating that trophic position itself is insufficient to explain the lower δ^{15}N of the cursorial spiders relative to the web spinners. This observation raises the possibility again that the web spinning process itself leads to δ^{15}N enrichment. However, further data are clearly needed to determine which of these explanations best accounts for the now recurring pattern of higher δ^{15}N in web-builders compared with cursorial taxa.

The patterns we found in δ^{13}C were less dramatic than in δ^{15}N, but matched expectations. Foliar samples consistently had the lowest δ^{13}C of all functional groups. Spider values were substantially offset from leaf values – approximately 4-5 per mill higher at all sites – which suggests a complex food chain consisting of many trophic levels below the spiders. This is plausible given that spiders are obligate predators (secondary consumers), and therefore must be trophically removed from plants by at least two levels. The conventional wisdom with δ^{13}C is "you are what you eat" (Hobson *et al.* 2010), meaning that most organisms are only slightly enriched in ^{13}C relative to their diets, with standard published offsets of less than 1 per mill for each successive trophic level (Post 2002). Indeed, meta-analysis of isotopic studies has found an average discrimination factor of ~0.3 per mill from one trophic level to the next within invertebrates (Caut *et al.* 2009), although it should be noted that many of the invertebrates included in that meta-analysis are aquatic, and no “standard” δ^{13}C offset for spiders or other terrestrial arthropods has yet been established. In an effort to fill the large gap between plants and spiders, we added samples of leaf litter from Upper Waiakea. The δ^{13}C of leaf litter fit neatly between leaves and spiders. This is to be expected given that the lighter ^{12}C is lost as respired $^{12}CO_2$ that is produced at a greater rate during decomposition, leaving the remaining litter relatively ^{13}C enriched (Dawson *et al.* 2002). Furthermore, this finding fits with previous studies of *Tetragnatha* trophic ecology, wherein it was observed that tipulid flies comprise a large proportion of the diet of *Tetragnatha* on Maui (Binford 2001, Blackledge *et al.* 2003). Because tipulid larvae often feed on decomposing leaves (Williams 1942), it is reasonable to surmise that tipulids' own δ^{13}C values fall close to those of the leaf litter, and that spiders on Hawaii Island become relatively enriched in their δ^{13}C composition when feeding on these insects.

Link between ecosystem properties and evolutionary processes

This study demonstrates that organisms at multiple tropic levels reflect the stoichiometric changes in soil across the geological chronosequence of the island, from very young (200-750 y) to older (20,000 y). The importance of this result is that it shows that the evolutionary processes associated with diversification are intimately linked to a landscape that, itself, changes through time. The detailed work of Vitousek, Shearer and Kohl (1989), Vitousek, Turner and Kitayama (1995), Vitousek *et al*. (1997) and Vitousek *et al*. (2009) documents the pattern of change in soil chemistry over extended time periods: Nitrogen and phosphorus increase almost linearly with time in the early stages of substrate development (up to 20,000 y for nitrogen and 150,000 y for phosphorus); this increase then levels off and declines on the oldest islands (4 my).

At the same time, it is now well established that organismal diversity increases over time during the early stages of formation of an island archipelago (Whittaker *et al.* 2008, Lim and Marshall 2017), and that higher trophic levels depend on lower levels in island community assembly (Simberloff and Wilson 1970), yet explanations for such patterns have, as yet, considered only area and age of the landscape. The documentation of peaks of diversity on middle aged islands of the Hawaiian Archipelago has been explained variously based on the interaction between age and area (Gillespie and Baldwin 2010, Lim and Marshall 2017). Notably missing from these studies is a link between evolutionary processes of diversification and shifts in nutrient availability associated with ecosystem succession. That organisms in Hawaii are intimately reflective of the ecosystem properties of their immediate habitat demonstrates that changes in nutrients associated with the island chronosequence are propagated through trophic and functional groups of entire biological communities. While initial work has begun to address the ecosystem consequences of evolutionary change (Elser *et al*. 2003, Laiolo *et al*. 2015), this study provides preliminary insights into how ecosystem change may affect processes of evolution.

Conclusions

Variation in δ^{15}N data indicates that different spider lineages reflect their different functional roles and trophic positions in Hawaiian food webs, from those feeding largely on primary consumers to those feeding exclusively on secondary and higher consumers. Importantly, the relationships between these groups, in terms of their δ^{15}N, remain strong across the chronosequence. Not only do the spiders' relative values of δ^{15}N show the same pattern at each site, but their isotopic signatures also reflect the availability of nitrogen at different sites from younger to older ecosystems. The tight relationship between N availability, plant isotopic values, and spider isotopic values strongly suggests that the spiders are dispersal-limited and their populations are isolated from one another, even across short distances. Such isolation may be an important mechanism of speciation within the *Tetragnatha* adaptive radiation. This study shows that these evolving lineages of spiders are intimately associated with the properties of their ecosystem, which is also changing. The tight connection between the organisms and the characteristics of their substrate highlights the importance of considering the role of soil properties, particularly chemistry, in addition to age and area, to understand how biodiversity accumulates over time.

Isotope	Effect	F	df	p-value
$\delta^{15}N$	site	692.1	2	**< 0.001**
	functional group	113.6	4	**< 0.001**
	site:functional group	8.615	6	**< 0.001**
$\delta^{13}C$	site	55.51	2	**< 0.001**
	functional group	95.15	4	**< 0.001**
	site:functional group	1.841	6	0.092

Table 1: Results of 2-way ANOVA testing for effects of site, functional group, and site x functional group interaction on stable isotopes of samples. Significant effects are indicated in bold.

Isotope	**Comparison**	**F**	**df**	**p-value**
$\delta^{15}N$	plants	78.74	2	**< 0.001**
	Spiny Leg	446.9	2	**< 0.001**
	web-builders	216.6	2	**< 0.001**
	Ariamnes	80.87	2	**< 0.001**
$\delta^{13}C$	plants	0.7997	2	0.482
	Spiny Leg	5.681	2	**0.005**
	web-builders	31.91	2	**< 0.001**
	Ariamnes	36.62	2	**< 0.001**

Table 2: Main effects of site (substrate ages: Upper Waiakea, 200-750 y; 'Ola'a, 2,100 y; Laupāhoehoe, 20,000 y) within each functional group of spiders and plants. Site was found to have a significant effect on both C and N isotope ratios of every functional group, with the exception of $\delta^{13}C$ in plants.

Isotope	**Site**	**F**	**df**	**p-value**
$\delta^{15}N$	Upper Waiakea (200-750 y)	68.38	4	**< 0.001**
	‘Ola’a (2,100 y)	34.23	3	**< 0.001**
	Laupāhoehoe (20,000 y)	28.90	3	**< 0.001**
$\delta^{13}C$	Upper Waiakea (200-750 y)	36.42	4	**< 0.001**
	‘Ola’a (2,100 y)	41.29	3	**< 0.001**
	Laupāhoehoe (20,000 y)	41.48	3	**< 0.001**

Table 3: Main effects of functional group within site. Functional groups were found to differ significantly from one another in their isotope ratios of both C and N at all three sites.

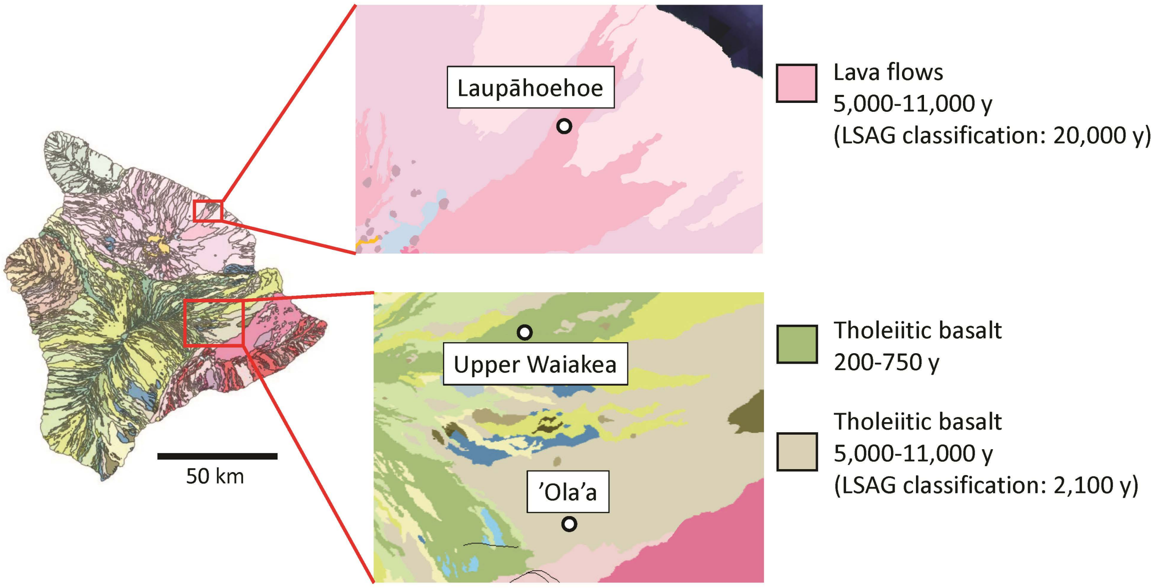

Figure 1: Map showing field sites where samples were collected. Colors represent geology and lava flow age, determined by United States Geological Survey (Sherrod et al. 2007). Where applicable, substrate age classifications determined by the Long Substrate Age Gradient (LSAG, Crews et al. 1995) are included.

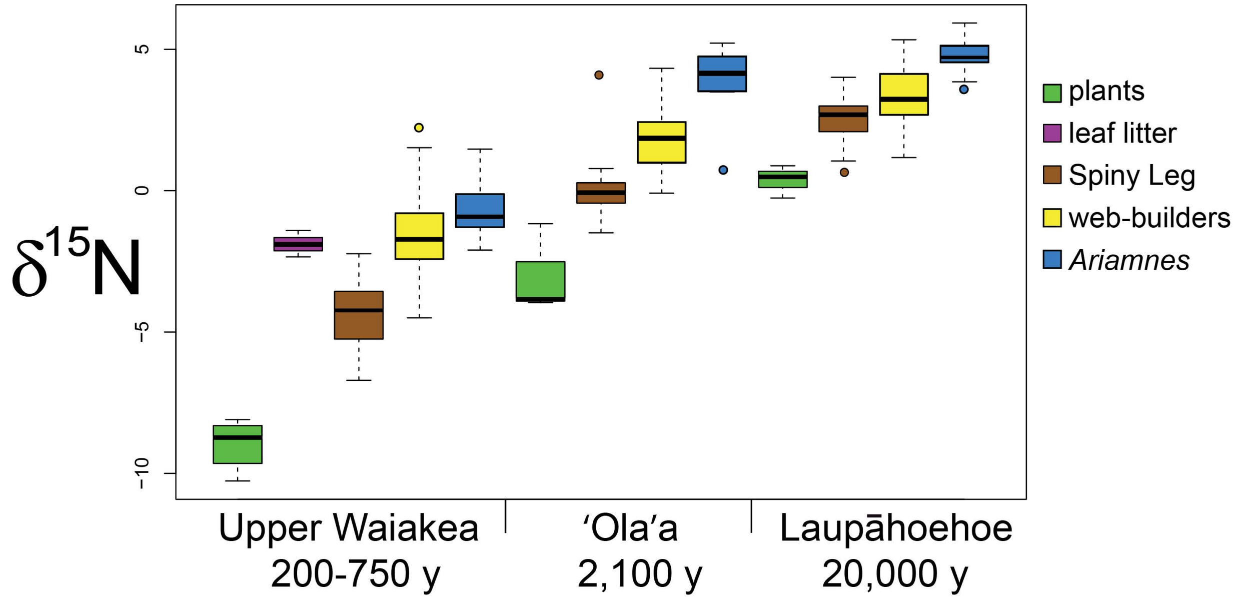

Figure 2: Nitrogen isotope ratio (δ^{15}N in ‰ units) of plant leaves (green), leaf litter (purple), Spiny Leg (brown), web-building (yellow) and *Ariamnes* (blue) spiders across sites of different ages.

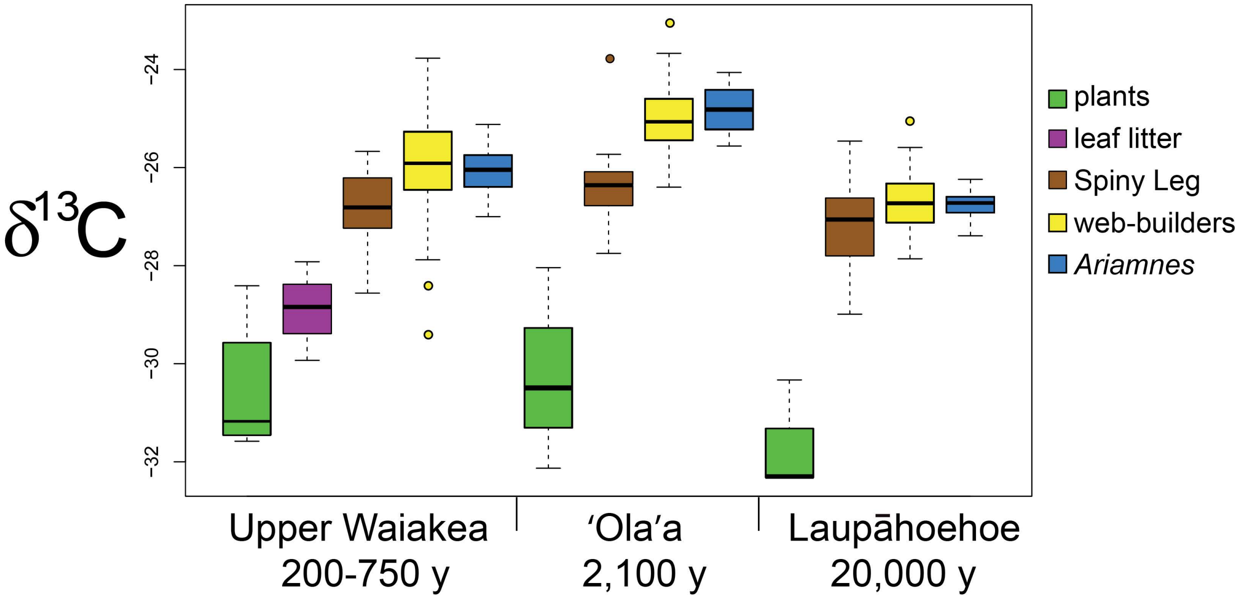

Figure 3: Carbon isotope ratio (δ^{13}C in ‰ units) of plant leaves (green), leaf litter (purple), Spiny Leg (brown), web-building (yellow) and *Ariamnes* (blue) spiders across sites of different ages.

Chapter 2

Spider webs, stable isotopes and molecular gut content analysis: multiple lines of evidence support trophic niche differentiation in a community of Hawaiian spiders

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Abstract

A pressing question in community ecology is how multiple closely related species are able to coexist. When these species occupy the same guild, positive and negative interactions between species such as competition for limited resources are expected to lead to niche partitioning. We tested for the possibility of niche partitioning in a community of Hawaiian *Tetragnatha* spiders, which are members of an adaptive radiation. Using nine sympatric *Tetragnatha* species belonging to two different clades - one web-building and the other actively hunting - we tested the hypothesis that trophic niches would differ significantly both 1) among species within a clade, indicating food resource partitioning, and 2) between the two clades, corresponding with their differences in foraging strategy. To assess trophic niches of the spider species, we measured a) web architecture (the structure of the hunting tool), b) site choice (the physical placement of the web in the habitat), c) stable isotope signatures of carbon and nitrogen in the spiders' tissues, and d) gut content of spiders based on metabarcoding data. Web architecture and isotopic data were used to generate niche hypervolumes for each species, and pairwise hypervolume overlaps were measured for each pair of species. We then tested for correlations between hypervolume overlap of web architecture and stable isotopes, and gut content beta diversity. We found significant differences in web architecture, site choice, and stable isotope signatures among the five web-building species, as well as significant isotopic differences between web-builders and active hunters. Gut content data also showed interspecific and inter-clade differences. Our results indicate trophic niche partitioning among species. However, no significant correlation was found between any pair of niche parameters. This suggests that additional, unknown, factors may be affecting web structure and isotopic signatures, and highlights the importance of examining multiple different types of data when characterizing the ecological niche of a species.

Introduction

Understanding the mechanisms by which numerous closely related co-occurring species can coexist remains a central goal in community ecology. One classic mechanism that promotes species coexistence is that of ecological niche partitioning, the process by which co-occurring populations divide available niche space by exploiting different sets of resources from each other (MacArthur 1958, Schoener 1974). Niche partitioning has been demonstrated in various ecological systems (Schluter and McPhail 1992, Losos *et al.* 2003, Losos 1994, von Rintelen *et al*. 2004). One important dimension by which niche partitioning is achieved is trophic (MacArthur 1958), and the trophic axis has been shown to be a primary driver in various vertebrate radiations, such as the anoles (Losos 2011), cichlid fish (Sage and Selander 1975) and Galápagos finches (Grant 1999). However, trophic niche differentiation has not received as much attention within invertebrate radiations (von Rintelen *et al*. 2004), probably because the relevant functional traits do not necessarily clearly manifest themselves morphologically. As such, the study of diet and trophic differentiation in insects and other arthropods necessitates a more holistic indirect approach.

Island habitats are particularly useful in such studies because they can provide relatively isolated (discrete) biological systems, in which the endemic biota have the opportunity to evolve novel phenotypes not seen in non-endemic relatives. Understanding the interacting roles of ecological and evolutionary processes in modulating these systems is a major frontier in biological science (Gillespie 2015). The Hawaiian Islands present an ideal venue for studies of ecology in an evolutionary context: this archipelago, deemed a "natural laboratory" (Simon 1987, Roderick and Gillespie 1998, Lim and Marshall 2017), has extraordinarily high levels of endemism due to its extreme isolation from other landmasses (> 4,000 km from the nearest mainland (Bennett and O'Grady 2013, Goodman *et al*. 2014)). Hence, multiple radiations have arisen on Hawaii (Carlquist *et al*. 2003, Givnish *et al*. 2009, Lerner *et al*. 2011, Lim and Marshall 2017). Among the arthropods, *Drosophila* flies (Magnacca and Price 2015), *Megalagrion* damselflies (Jordan *et al*. 2003), *Nesophrosyne* leafhoppers (Bennett and O'Grady 2013), and *Tetragnatha* spiders (Gillespie 2015) are well established examples. In each instance, representatives of the lineage have evolved into various new phenotypes not found in their mainland counterparts. Often, several species within a radiation coexist within the same habitat, raising the question of whether niche partitioning is occurring – in other words, whether these novel phenotypes correspond to the exploitation of new ecological niches.

Here, we focus on the potential role of trophic differentiation in a radiation of island *Tetragnatha* spiders. *Tetragnatha*, the "long-jawed orb-weavers," are globally distributed and largely similar in morphology and ecology (Kaston 1978). Most *Tetragnatha* have long, slender bodies and long chelicerae (jaws), and spin horizontal orb webs near bodies of water. On the Hawaiian archipelago, however, they have diversified and evolved a myriad of novel morphologies and behaviors, including the loss of web-building behavior in one clade (Gillespie 1991, 2002b). In contrast with the stereotyped morphology of most mainland *Tetragnatha*, the Hawaiian species have evolved new morphologies (e.g. stouter bodies, shorter jaws, humps or tubercles on the abdomen in some species) and a variety of colorations, from brown to maroon to bright green to red, these particular appearances believed to reflect adaptations to camouflaging on different types of substrate during the day (Gillespie 2004).

There are over 30 described species of *Tetragnatha* on Hawaii (Gillespie 1991, 1992, 1994, 2002b, 2003), and multiple species often co-occur locally. As such, these spiders represent a suitable system in which to probe the possible role of trophic differentiation in species co-existence at the community scale. The diet of spiders is a promising potential axis of divergence because food resources - insects and other arthropods - are expected to be somewhat limited and irregular or seasonal in their availability (Wolda 1988). However, dietary studies in spider systems present a challenge because 1) predation events are rarely observed; 2) *Tetragnatha* webs are fragile and are therefore usually destroyed in the process of intercepting prey (Lesar and Unzicker 1978); 3) even when webs withstand the collision of prey, animals that have been trapped are not necessarily consumed by the spiders. In addition, morphological gut content analysis is not feasible because spiders ingest food in liquid form.

We addressed these challenges by using four types of data to characterize the trophic niches of spiders and to evaluate the degree to which the species divide the available resources (food) in the habitat. We first measured spiders' foraging behavior by looking at both 1) web architecture, the structure of the spiders' foraging tool, and 2) site choice, the physical placement of webs in the habitat. Both web architecture and site choice are likely to determine the types of prey that spiders can catch: different web forms may be more effective at intercepting particular types of insects (Harmer *et al*. 2015, Blackledge and Zevenbergen 2006, Sensenig *et al*. 2013), and web placement in a given microhabitat should result in the capture of prey that occur within that microhabitat. Additionally, we analyzed spiders' lifelong trophic niche by measuring the ratios of stable carbon and nitrogen isotopes in the spiders' tissues. Stable isotopes are a well established tool in food web studies (Peterson *et al*. 1985, Cabana and Rasmussen 1994, France 1995). Both nitrogen and carbon isotopes are useful for assessing trophic position, as the ratio of heavier to lighter isotope (δ^{13}C or δ^{15}N) tends to increase in a stepwise pattern with successive trophic levels (Post 2002). Finally, we identified the gut contents of spiders to the order level, using a metabarcoding approach.

We focused on a community of nine co-occurring *Tetragnatha* species in a native montane forest of East Maui, which contains some of the highest species richness among the Hawaiian Islands. Five of the species are web-builders; the remaining four are active hunters in the Spiny Leg clade of *Tetragnatha*, all of whose members hunt without the use of a web (Gillespie 1991). Three of the five web-building species – *T. eurychasma*, *T. stelarobusta* and *T. trituberculata* – are very closely related, members of the "Eastern Forest" clade (Kennedy *et al.* in prep(b)). Despite this close relatedness, these three and the other two web-building species differ visibly from one another in their morphology. *T. acuta* is cream-colored to brown, with a single hump on the dorsal midpoint of its abdomen; *T. eurychasma* is small and green with an oval abdomen; *T. filiciphilia* is small and bright yellow to chartreuse with a distally tapered abdomen; *T. stelarobusta* is the largest of the five species, brown in color with a cigar-shaped body; and *T. trituberculata* is stout-bodied, with a green-and-black-mottled abdomen rising to a hump in the middle with three distinctive tubercles (Gillespie 1992, 1994). Among the four Spiny Leg species, three of four previously established morphological "ecotypes" (Gillespie 2004) are represented: *T. brevignatha* and *T. waikamoi* belong to the "green" ecotype, *T. kamakou* to the "maroon" ecotype, and *T. quasimodo* to the "large brown" ecotype. These ecotypes are associated with particular substrate types, which afford effective camouflage based on the spiders' coloration (Gillespie 2004).

Earlier observational work suggests that dietary differences may exist among sympatric web-building *Tetragnatha* species (Blackledge *et al*. 2003) and, on a broader scale, between the web-building spiders and the actively hunting Spiny Leg spiders (Binford 2001). Furthermore, it was recently found that web-building *Tetragnatha* carry a different isotopic signature in their tissues from co-occurring Spiny Leg *Tetragnatha* on the Big Island of Hawaii (Kennedy *et al*. 2018). We therefore hypothesize that trophic niche partitioning occurs among sympatric *Tetragnatha* in East Maui, and that dietary niche is significantly different between web-builders and Spiny Leg spiders due to the fundamental difference in hunting style between these two groups. We predict that 1) web-building spiders will show significant interspecific differences in web structure, site choice, and C and N stable isotope signatures; and 2) according to hunting strategy, there will be differences in both stable isotope signatures and taxonomic composition of gut contents between the web-builders and the Spiny Leg spiders.

Methods

Study system

Sampling took place in The Nature Conservancy's Waikamoi Preserve (East Maui), located on the northwestern (windward) slope of Haleakalā Volcano. Five species of web-building *Tetragnatha*, all endemic to the Maui Nui complex (comprised of the islands of Maui, Molokai, Lanai and Kaho'olawe), occur at our sites: *T. acuta*, *T. eurychasma*, *T. filiciphilia*, *T. stelarobusta* and *T. trituberculata*. Four Spiny Leg species are also found at these sites: *T. brevignatha*, *T. kamakou*, *T. quasimodo* and *T. waikamoi*. These spiders display clear interspecific differences in size, shape and coloration (Gillespie 1991, 1992, 1994), making them easily identifiable in the field.

Web-building *Tetragnatha* spin two-dimensional orb webs. A spiral of sticky silk, the "capture spiral," is supported by several spokes, or "radii," which converge into a central hub (Fig. 1). The section of web between two adjacent radii is called a "sector." *Tetragnatha* spiders are nocturnal, and generally spin a fresh web every night, then dismantle and eat the web (recycling the silk) every morning. These webs are relatively flimsy and sustain significant damage when they intercept prey, so spiders also generally dismantle their webs after each prey capture event.

Sample collection

We conducted sampling in June and July 2013, June 2014, November 2016, and June 2017, at two different elevations: low (Maile Trail, 1380 – 1500 m) and high (Waikamoi Boardwalk, 1800 – 1950 m). Because off-trail access was restricted in order to protect fragile endemic plants, we performed searches along the trail rather than following straight transects through the forest. For each web found, we collected the spider into a clean snap cap vial. We then dusted the web with cornstarch (to enhance visibility) and photographed the web from a 90° angle with a ruler held in frame for scale. The digital photos served as our samples for web architecture analysis. To evaluate microhabitat use (i.e. the physical placement of webs in the habitat), we recorded the vegetation type (tree, shrub, fern, vine or grass) to which the web was attached, and measured the web's height above the ground and angle from vertical (0° = vertical, 90° = horizontal). These measurements served to describe the spider's site choice. Spiny Leg spiders, which do not build webs, were collected directly from the vegetation into individual snap cap vials.

Web measurements

We collected data on a total of 188 webs (*T. acuta*: n = 36, *T. eurychasma*: n = 39, *T. filiciphilia*: n = 57, *T. stelarobusta*: n = 43, *T. trituberculata*: n = 13). We measured webs from digital photos using ImageJ (National Institutes of Health, USA). For each web, we took the measurements summarized in Table 1 (see also Fig. 1). Capture thread length (CTL), the total length of sticky capture spiral, was estimated using the large-scale formula of Heiling *et al*. (1998):

$$CTL = \Sigma((T_l + T_s) / 2) * x_i$$

where x_i is the number of rows of capture thread in a sector, T_l is the length of outermost (longest) row of capture thread in a sector, and T_s = length of innermost (shortest) row of capture thread in a sector.

Mesh width (MW), the mean distance between adjacent rows of capture spiral, was estimated using the following formula (Blackledge *et al*. 2003):

$$MW = 0.5 * (D_u / (R_u - 1) + D_b / (R_b - 1))$$

where D_u is the length of a designated "upper" sector (i.e. chosen from the upper half of the web) from innermost to outermost row of capture thread; R_u is the number of rows of capture thread in D_u; D_b is the length of a designated "lower" sector; and R_b is the number of rows of capture thread in D_b.

Capture area of webs was measured directly from the image, and included only the area of the web that was covered by sticky silk. Radii, the stiff spokes supporting the capture thread, were counted from the photos, and the mean number of rows of sticky silk was calculated for each sector. Using the primary measurements of capture thread length, mesh width and capture area, we calculated two metrics of silk density: silk density 1 = capture thread length / capture area, and silk density 2 = capture area / mesh width. Together, these metrics (summarized in Table 1) comprise web architecture.

Stable isotope analysis

Spider specimens collected in 2014 and 2016 were used for C and N stable isotope analysis (*T. acuta*: n = 26, *T. eurychasma*: n = 26, *T. filiciphilia*: n = 10, *T. stelarobusta*: n = 25, *T. trituberculata*: n = 11, *T. brevignatha*: n = 10, *T. kamakou*: n = 9, *T. quasimodo*: n = 10, *T. waikamoi*: n = 10). Each spider collected for isotopic analysis was kept in a plastic snap cap vial and euthanized in a freezer. Individuals were then photographed up close using a Nikon D5200 with a macro lens, creating a digital voucher. The spiders were air-dried separately at room temperature. Clean beads of silica gel were added to each vial to accelerate drying and prevent decomposition of the spiders.

We homogenized individual spiders with a mortar and pestle and weighed each sample into a 9 x 5 mm tin capsule, aiming for 1.5 mg of tissue in order to optimize the efficiency of the analysis based on the C:N ratio in spiders' tissues (S. Mambelli, pers. comm., 2014). When spiders weighed less than 2.5 mg, we used whole bodies to prevent excessive loss of material in the grinding process. We conducted stable isotope analyses at the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. Samples were analyzed for carbon and nitrogen content (percent dry weight) and carbon and nitrogen stable isotope ratios by continuous flow dual isotope analysis on a CHNOS Elemental Analyzer (vario ISOTOPE cube, Elementar, Hanau, Germany) connected to an IsoPrime100 mass spectrometer (Isoprime Ltd, Cheadle, UK).

Stable isotope ratios are expressed in "delta" notation (‰). The isotopic composition of a sample relative to that of a standard is measured as a per mill deviation and written as $\delta^{13}C$ (or $\delta^{15}N$) = ($R_{sample}/R_{standard}$ - 1) x 1,000, where R is the ratio of heavy to light isotopes. The standard used for carbon was Vienna Pee Dee Belemnite, and the standard for nitrogen was air. The reference material NIST SMR 1547 (peach leaves) was used as a calibration standard. Long-term external precision based on reference material NIST 244 SMR 1577b (bovine liver) is 0.10‰ for carbon and 0.15‰ for nitrogen isotope analyses.

Gut content analysis

Gut content specimens were collected directly into 95% ETOH and kept frozen at -20° until DNA extraction. Because most of a spider's digestive tract is located in its opisthosoma (abdomen), we made DNA extractions from whole opisthosomas. DNA was extracted using the Qiagen Gentra Puregene kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, with a 6-hour cell lysis and a 30-minute incubation with RNase A to remove RNA. PCR was used to amplify our gut content marker, a 211-bp fragment of mitochondrial Cytochrome Oxidase I (COI) that partially overlaps with the barcoding region, using the primers ZBJ-ArtF1c (AGATATTGGAACWTTATATTTTATTTTTGG) and ZBJ-ArtR2c (WACTAATCAATTWCCAAATCCTCC) (Zeale *et al*. 2011). We chose this short DNA marker because we expected the spiders' prey to be mostly digested within their guts, and therefore the prey DNA should be broken down into small fragments. Furthermore, this marker was previously found to be effective in gut content analysis of spiders (Krehenwinkel *et al*. 2017) and has the added benefit that it amplifies poorly in *Tetragnatha* spp., which means it amplifies as much or more prey DNA than the DNA of the spider itself. This is an important consideration in molecular gut content analysis because the predator's own DNA often vastly outweighs the prey DNA, requiring very high sequencing coverage to obtain sufficient yield of prey DNA (Piñol *et al*. 2014, Toju and Baba 2018). PCR cycling conditions were as follows: initial denaturation at 95° for 15 minutes; 31 cycles of denaturation at 94° for 30 seconds, annealing at 46° for 90 seconds, and extension at 72° for 90 seconds; and final extension at 72° for 10 minutes. Both forward and reverse primers had Illumina TruSeq tails attached to the 5' ends. These tails served as binding sites for barcoding primers, which were added to the products in a second round of PCR so that each specimen was labeled with a unique combination of forward and reverse barcodes.

After the second round of PCR, samples were visualized on a 1.5% agarose gel and pooled together in equal amounts based on gel band intensity. The pool was diluted to 4 nM concentration and run on a MiSeq with V3 chemistry at UC Berkeley's QB3 (Berkeley, CA) and at the California Academy of Sciences' Center for Comparative Genomics (San Francisco, CA).

Sequences were demultiplexed by barcode combination, assembled with PEAR (Zhang *et al*. 2014), and quality filtered and converted to FASTA format with FastX Toolkit (Gordon and Hannon 2010). Sequences were then clustered into OTUs (operational taxonomic units) at 97% similarity, and chimeras were removed, using USearch (Edgar 2010). Taxonomy of prey OTUs was identified to the order level using a BLAST search (Altschul *et al*. 1990) against the National Center for Biotechnology Information (NCBI) database.

Data analysis

We performed principal component analysis (PCA) on web architecture variables (capture area, capture thread length, mesh width, number of radii, number of rows, and the two metrics of silk density; Table 1) in R v 3.5.0 (R Core Development Team 2018). Capture area and capture thread length were first log-transformed so that they would be normally distributed. Variables were scaled to unit variance before multivariate analysis so that variable loadings would be more directly comparable. After testing our data for any violations of assumptions of normality, we performed a one-way ANOVA to test for differences among species along axes PC1 and PC2, followed by a post-hoc Tukey's Honest Significant Difference (HSD) test while controlling for multiple comparisons.

To test for differences in site choice (web attachment microhabitat), we performed a non-parametric Fisher's exact test on the number of times each species had been observed building webs on different vegetation groups (fern, tree, vine, grass or shrub). For the two quantitative variables composing site choice (height and angle of webs), we performed a one-way ANOVA followed by a Tukey's HSD test to identify pairwise differences among species.

To test for species-specific differences in δ^{15}N and δ^{13}C, we performed a one-way ANOVA, followed by post-hoc Tukey's Honest Significant Difference (HSD) test to control for multiple comparisons. In addition, to test for broader-level differences in isotopic signature between web-building and Spiny Leg clades, we performed a one-way ANOVA between web-builders and Spiny Leg spiders for both δ^{15}N and δ^{13}C.

Testing for relationships among niche parameters

To determine whether a correlation exists among 1) web architecture and isotopic signature, 2) web architecture and gut content, or 3) isotopic signature and gut content, we calculated species pairwise differences in web architecture, isotopic signature and gut content. We first quantified the variability in web architecture and isotopic composition occupied by each species by constructing multi-dimensional hypervolumes for both web and isotope variables. This approach quantifies the multi-dimensional web or isotope space occupied by each species using multivariate density kernel estimation (Blonder *et al*. 2014). This is an improvement over convex hull methods, which are more sensitive to outlier points. Hypervolumes were calculated using the 'hypervolume' package in R (Blonder *et al*. 2014). Web hypervolumes were constructed using loadings for the first three PCA axes, as hypervolume calculations can be sensitive to collinear variables. Isotope hypervolumes were calculated using δ^{13}C and δ^{15}N values. For gut content data, we calculated the beta diversity (Bray-Curtis distance) between pairs of species.

We then calculated pairwise differences in web and isotope hypervolumes by computing the pairwise Sorensen overlap between species hypervolumes. For two hypervolumes, A and B, hypervolume Sorensen overlap is calculated as follows: $2*(A \cap B) / (A + B)$. Values range from 0 (i.e., species are completely dissimilar in web architecture or isotope composition) to 1 (species are completely similar in web architecture or isotope composition). If web architecture were driving differences in isotopic signatures through a selective effect on diet, one would expect species with more similar webs to be more similar in isotopic composition (i.e., web hypervolume overlap should be positively correlated with isotope hypervolume overlap). We assessed the relationship between web hypervolume overlap and isotope hypervolume overlap using a Pearson's correlation test.

For gut content data, we first rarefied the prey read counts of each specimen to equal coverage (500 reads per specimen) using GUniFrac (Chen *et al.* 2012), and randomly subsampled the data to a sample size corresponding to the least represented species (n = 5 for web-building species, n = 10 for Spiny Leg species) in order to obtain even sampling. We then assessed the degree of pairwise dissimilarity in diet by calculating the Bray-Curtis distance between pairs of species. Using the hypervolume overlaps calculated as described above, we then tested for correlations among 1) gut content and web architecture and 2) gut content and isotopic signature, using a Pearson's correlation test.

Results

Web architecture

The first two axes in our principal component analysis of web architecture account for 87.3 % of the total variation in the sample population (Fig. 2). The first axis (PC1), accounting for 51.9 % of variation, is positively correlated with number of rows, followed closely by number of radii, capture thread length and silk density 2. Mesh width is negatively correlated with PC1, but has a weaker association than the other variables. Thus, the first axis generally describes web density: as spiders increase in their PC1 value, their webs have longer capture threads, more silk per unit area, larger numbers of radii, and smaller mesh widths. The second PCA axis (PC2, 35.4 % of variation) is positively associated with silk density 1 (capture thread length divided by capture area). PC2 is negatively associated with capture area and mesh width. Thus, as spiders increase in PC2 value, their webs are smaller and denser in silk, with shorter distances between adjacent rows of capture spiral.

We found significant interspecific differences in species position on PC1 (F = 34.87; d.f. = 4,183; $p < 0.05$) and PC2 (F = 46.79; d.f. = 4,183; $p < 0.05$)(Fig. 3). *T. stelarobusta* was significantly higher in PC1 (web density) than all other species, followed by *T. filiciphilia*. *T. acuta* and *T. eurychasma* had the lowest PC1 values. For PC2, *T. filiciphilia* had significantly higher values (i.e. smaller and denser webs) than the other species, and *T. trituberculata* had significantly lower values (larger and less dense webs) than all species except *T. eurychasma*.

Site choice

Spider species had non-random differences in the vegetation group on which their webs were built (Fisher's exact test, χ^2 = 103.02, 2000 replicates, $p < 0.005$)(Fig. 4). *T. trituberculata* was mostly found on trees (*Myrsine*, *Melicope*), and to a lesser extent on shrubs (*Vaccinium*, *Rubus*, *Leptecophylla*). *T. stelarobusta* occurred mostly on ferns (*Dryopteris*, *Diplazium*, *Sadleria*) and shrubs. *T. filiciphilia* was the only species frequently found on vines, specifically *Alyxia oliviformis*. *T. acuta* and *T. eurychasma* both occurred mainly on shrubs and less so on ferns.

Angle and height of webs also differed significantly among species (angle: F = 17.57, d.f. = 4, 183, $p < 0.005$; height: F = 12.11, d.f. = 4,183, $p < 0.005$)(Fig. 5). *T. trituberculata* built significantly more vertical webs (i.e. with angles closest to 0°) than others (mean = 13°), followed by *T. acuta* and *T. stelarobusta*. *T. filiciphilia* and *T. eurychasma* built obliquely oriented webs, and *T. filiciphilia* had the greatest deviation from vertical (mean = 50°). *T. trituberculata* built webs significantly higher than all other species (mean = 186 cm), while *T. stelarobusta* built webs significantly lower than all others (mean = 109 cm).

Isotopic differences among species

There were significant differences in δ^{15}N (ANOVA F = 15.01; df = 4,93; $p < 0.05$) and δ^{13}C (ANOVA F = 5.50; df = 4,93; $p < 0.05$) among web-building *Tetragnatha* species (Fig. 6). Using a post-hoc Tukey's Honest Significance Differences test, we found that *T. filiciphilia* and *T. stelarobusta* were both significantly higher in δ^{15}N than the other three web-building species ($p < 0.05$). *T. stelarobusta* had significantly lower δ^{13}C values than all other web-building species except for *T. eurychasma*. *T. eurychasma* had significantly lower δ^{13}C values than *T. trituberculata* and *T. filiciphilia*.

Within Spiny Leg species, *T. quasimodo* was significantly lower than its congeners in δ^{15}N (ANOVA F = 8.24, d.f. = 3,35, $p < 0.001$) and significantly higher than either *T. brevignatha* or *T. kamakou* in δ^{13}C (ANOVA F = 5.90, d.f. = 3, 35, $p = 0.002$)(Fig. 7). We also performed a broader, clade-level comparison of isotopic signatures between the web-builders and the Spiny Leg spiders. We found a highly significant difference between web-builders and Spiny Leg spiders for both δ^{15}N (ANOVA F = 50.41 d.f. = 1,135, $p < 0.001$) and δ^{13}C (ANOVA F = 37.24, d.f. = 1,135, $p < 0.001$)(Fig. 8), with web-builders falling higher than Spiny Leg spiders for both isotopic signatures.

Gut content

Based on the numbers of sequences obtained from spiders' gut contents, each species showed a distinctive prey composition at the order level (Fig. 9). Among the web-builders, *T. acuta* and *T. eurychasma* had mostly Diptera (64% of total reads for *T. acuta*, 75% for *T. eurychasma*) and moderate amounts of Hemiptera (30% for *T. acuta*, 15% for *T. eurychasma*), while the remaining three web-building species contained a more even representation of prey orders. *T. filiciphilia* had a large proportion of Lepidoptera (49%) and Diptera (29%), as well as being the only species found with Psocoptera in its gut (17%). *T. stelarobusta* also contained a relatively high proportion of Lepidoptera (34%) and Diptera (43%), with moderate contributions of Araneae (11%) and Collembola (9%). *T. trituberculata* had mostly Lepidoptera (35%), Hemiptera (31%) and Diptera (29%). Among the Spiny Leg species, *T. brevignatha* had the highest proportion of Diptera (39%). *T. brevignatha* also had a relatively even representation of Lepidoptera (20%), Araneae (18%), and Hymenoptera (14%). *T. kamakou* contained the highest order richness of prey, with seven orders found in its gut extracts (42% Lepidoptera, 28% Hemiptera, 12% Hymenoptera, 8% Diptera, 5% Coleoptera, 3% Araneae, and 2% Acari). *T. quasimodo* had mostly Hemiptera (42%) and Lepidoptera (35%), as well as some Diptera (16%). *T. waikamoi* had fairly even representation of its prey, with moderate to high contributions of Diptera (29%), Hymenoptera (26%), Lepidoptera (20%), and Hemiptera (15%), as well as some Collembola (8%). On the whole, web-builders tended to have more Diptera while Spiny Leg spiders had more Hemiptera and Hymenoptera in their guts, and both clades had substantial contributions of Lepidoptera (Fig. 9).

Bray-Curtis distances showed moderate to high complementarity between most pairs of spider species within each clade (Tables 2 and 3), ranging from approximately 0.2 – 0.5. In some species, intraspecific Bray-Curtis distances were also relatively high. *T. stelarobusta*, for example, had a higher intraspecific Bray-Curtis distance (0.302) than its distance from *T. eurychasma* (0.279), and an approximately equal intraspecific distance to its distance from *T. trituberculata* (0.301). *T. acuta* had a larger distance from itself (0.234) than from *T. trituberculata* (0.207). The intraspecific Bray-Curtis distance of *T. kamakou* (0.232) was approximately equal to its distance from *T. waikamoi* (0.235), and likewise the intraspecific distance of *T. quasimodo* (0.296) was close to the distance between *T. quasimodo* and *T. waikamoi* (0.301). All other species had higher Bray-Curtis distances from other species than from themselves.

Correlations among niche parameters

Pairwise overlaps of web architecture hypervolume and isotope hypervolume, and pairwise Bray-Curtis distances of gut content data, all showed trends toward being correlated with one another, but no significant correlation was found. Web architecture overlaps were slightly, but not significantly, positively correlated with stable isotope overlaps (Pearson's product moment correlation coefficient = 0.521, p = 0.122)(Fig. 10). A non-significant negative correlation was found between web architecture overlap and gut content dissimilarity (Pearson's coefficient = -0.592, p = 0.071). In web-builders, a non-significant trend toward negative correlation was found between isotope hypervolume overlap and gut content dissimilarity (Pearson's coefficient = -0.522, p = 0.121)(Fig. 11). In Spiny Leg spiders, there was a negative but non-significant relationship between isotope hypervolume overlap and gut content dissimilarity (Pearson's coefficient = -0.777, p = 0.069).

Discussion

We found significant differences in web architecture, site choice, and stable isotope signatures of *Tetragnatha* spider species in East Maui. These species-specific differences in web form (foraging strategy) and isotopes (lifelong trophic niche) suggest resource partitioning. This is supported by previous observations of *Tetragnatha* feeding behavior and web structure (Binford 2001, Blackledge *et al*. 2003, Blackledge and Gillespie 2004). Our study provides a robust, multidimensional picture of trophic niche in the five web-building species by integrating aspects of behavior (web structure and site choice) in addition to chemical signatures of the spiders' diets (stable isotopes) and gut content.

Niche partitioning may be accomplished through divergence in web architecture and site choice, as these traits can influence the assemblage of prey captured by a spider. For example, the araneid orb weaver *Parawixia bistriata* alters its web structure at different times of day, apparently specializing on certain prey types that fly at different times (flies in the night, termites in the daytime)(Sandoval 1994). Mesh width has been found to potentially influence prey retention times in the orb-weaving spider *Argiope aurantia*, which suggests that by adjusting their silk density, spiders can optimize webs to retain stronger (larger or more energetic) prey for longer, giving the spider time to deliver the killing bite (Blackledge and Zevenbergen 2006). The giant orb-weaver *Nephila pilipes* can alter its mesh width, as well as biomechanical properties of its silk, in response to being fed different prey types (Tso *et al*. 2007). Site choice can also affect the types of prey that are typically intercepted by a web. Sheet web weavers (Linyphiidae) have been observed building webs preferentially in microhabitats that are rich in aphids, thysanopterans and collembolans, their typical prey, relative to other potential web-building sites (Harwood *et al*. 2003). *Argiope aurantia* captures different orders of insects depending on the type of vegetation, grass versus shrub, on which it builds its web (McReynolds 2000). In the same way, it is plausible that Hawaiian *Tetragnatha* select web-building sites that improve their chances of capturing particular prey types (e.g. herbivorous insects associated with specific host plants, or flies that forage in the canopy versus the understory), while being constrained in their site choice by the presence of congeners in the available habitat. The significant species effect we found for both web structure and site choice suggests that trophic niche partitioning is occurring on the species level in this group of spiders.

The interpretation of our isotope data is somewhat complex due to the potential contributions of different variables. The differences in δ^{13}C among species, with *T. trituberculata* and *T. filiciphilia* being highest and *T. stelarobusta* and *T. eurychasma* lowest, may be related to diet, but could also reflect subtle microhabitat differences such as variations in sun exposure (Zimmerman and Ehleringer 1990, Li *et al.* 2007) or humidity (Liu *et al.* 2003, Porter *et al*. 2009). Most isotopic studies find that δ^{13}C closely reflects the signature of an animal's food source, and changes little from one trophic level to the next (Hobson *et al*. 2010). If this is the case in the Hawaiian *Tetragnatha*, then it is plausible that isotopic signatures of the prey's microhabitat – e.g. sun-exposed canopy versus darker, more humid understory vegetation – will be borne up into the spiders' tissues, even if the taxonomic composition of prey is similar. The ambiguity of our δ^{13}C results calls for further study on the isotopic signatures of putative prey from a variety of different microhabitats in order to disentangle these potential effects.

The differences we found in δ^{15}N – *T. filiciphilia* and *T. stelarobusta* falling significantly higher than the others – may indicate species-specific dietary niches. Specifically, higher values of δ^{15}N are associated with higher trophic level, with standard discrimination rates generally agreed to be approximately 3.4 per mill with each successive trophic level (Post 2002). Thus, it is possible that higher-level predators, such as predatory flies (e.g. Dolichopodidae), parasitoid flies or wasps, make up a greater proportion of the diets of *T. stelarobusta* and *T. filiciphilia* than of the other three species. Although we have taxonomic data on these spiders' gut contents, at the moment it is difficult to obtain reliable data at taxonomic levels lower than order because many taxa are not represented in the NCBI reference database. Ecological analyses relying upon gut content could be substantially improved in their precision and resolution by the availability of sequences representing a broader range of taxa.

However, diet is not necessarily the only factor affecting the stable nitrogen isotope ratios in spiders' tissues. Alternatively, the higher δ^{15}N in *T. stelarobusta* and *T. filiciphilia* may be related to the higher density of silk in these two species' webs: *T. filiciphilia* fell highest for "Silk Density 1" (Capture Thread Length / Capture Area), and *T. stelarobusta* highest for "Silk Density 2" (Capture Area / Mesh Width). It may be that the act of spinning and recycling (eating) silk contributes to the loss of the lighter ^{14}N isotope and ultimately the higher concentration of ^{15}N in the spider's tissues. If this is the case, then this phenomenon could at least partially account for the higher δ^{15}N that we found in web-builders when compared with Spiny Leg spiders. While very little is known about the impact of silk spinning and recycling on isotopes, other studies have found silk-recycling web spinners to fall at higher values of δ^{15}N than those that hunt without webs or do not recycle their silk (Sanders *et al*. 2015). Indeed, a recent study of Hawaiian *Tetragnatha* on the Big Island of Hawaii found the same pattern of higher δ^{15}N in web-builders than in members of the Spiny Leg clade (Kennedy *et al*. 2018). While this is an intriguing pattern, more rigorous testing is needed to determine whether the silk recycling act itself accounts for these differences in nitrogen isotopic signature in spiders' tissues.

Our findings build on earlier studies of *Tetragnatha* diet (Binford 2001, Blackledge *et al*. 2003). These studies found general trends in the taxonomic assemblage of prey observed in the jaws or on the webs of the spiders, suggesting broad dietary differences. Blackledge *et al*. (2003) found a greater proportion of tipulids (order Diptera) than other taxa in the webs of *T. eurychasma*, suggesting that the relatively large mesh width in this species may be well suited to intercepting the slender-bodied tipulids. Our own findings show Diptera occupying a very high proportion – 75% – of the diet of *T. eurychasma*, although we are unable to ascertain how much of this prey (if any) is made up of tipulids as opposed to other dipterans. *T. stelarobusta* has been recorded catching mostly lepidopterans (Binford 2001, Blackledge *et al*. 2003); Blackledge *et al*. (2003) suggested that the relatively high silk density of *T. stelarobusta* could facilitate the capture of moths, which might otherwise escape a loosely woven web by shedding scales from their wings. Indeed, our gut content data show a high proportion of Lepidoptera in the diets of our two species that spin the most silk-dense webs: *T. filiciphilia* (49%) and *T. stelarobusta* (34%). Although some studies exist on the mechanistic features and prey-capture efficiency of orb webs (Rypstra 1982, Sherman 1994, Venner and Casas 2005, Blackledge and Zevenbergen 2006, Sensenig *et al*. 2012, 2013), there is clearly a need for more of these studies, on a broader variety of systems, to address the intriguing question of exactly how the structure of a spider's web affects its function as a hunting tool.

Conclusions

Overall, our results suggest that the evolutionary divergence of the Hawaiian *Tetragnatha* may be associated with trophic niche differentiation – both at broader (web-builders versus active hunters) and at finer (interspecific) scales. Whether such dietary differentiation acted as a driver for the spiders' evolution, or occurred after their genetic divergence, is unknown. However, given the apparent behavioral and ecological differences among these species, we find support for the hypothesis that trophic niche differences serve as a mechanism of species coexistence within spider communities.

Our study provides a comprehensive picture of dietary niche, and overcomes the challenges inherent in traditional methods of direct observation for dietary analysis of predators. By integrating four types of data – web architecture, site choice, stable isotopes and gut content – we analyze niche across multiple dimensions, incorporating information on spiders' recent meals (gut content), lifelong dietary niche (stable isotopes), and hunting behavior (web architecture and site choice). That these different niche parameters were not significantly correlated with one another suggests a high level of ecological complexity, and points to the importance of including multiple different types of data in analyses of species interactions.

Web architectural trait	**Description**
Capture thread length = CTL (cm)	Total length of sticky capture spiral in the web
Capture area = CA (cm^2)	Two-dimensional area covered by capture spiral
Mesh width = MW (cm)	Mean distance between two adjacent rows of capture spiral
Radii	Number of supporting spokes (radii)
Rows	Mean number of rows of capture spiral
Silk density 1 = SD1 (cm / cm^2)	CTL / CA
Silk density 2 = SD2 (cm)	CA / MW

Table 1*:* Definitions of web architecture metrics.

	T. acuta	*T. eurychasma*	*T. filiciphilia*	*T. stelarobusta*	*T. trituberculata*
T. acuta	0.234	NA	NA	NA	NA
T. eurychasma	0.243	0.175	NA	NA	NA
T. filiciphilia	0.477	0.446	0.317	NA	NA
T. stelarobusta	0.333	0.279	0.410	0.302	NA
T. trituberculata	0.207	0.244	0.343	0.301	0.069

Table 2: Pairwise Bray-Curtis dissimilarities in gut content in web-building *Tetragnatha.*

	T. brevignatha	*T. kamakou*	*T. quasimodo*	*T. waikamoi*
T. brevignatha	0.336	NA	NA	NA
T. kamakou	0.372	0.232	NA	NA
T. quasimodo	0.432	0.318	0.296	NA
T. waikamoi	0.390	0.235	0.301	0.215

Table 3*:* Pairwise Bray-Curtis dissimilarities in gut content in Spiny Leg *Tetragnatha.*

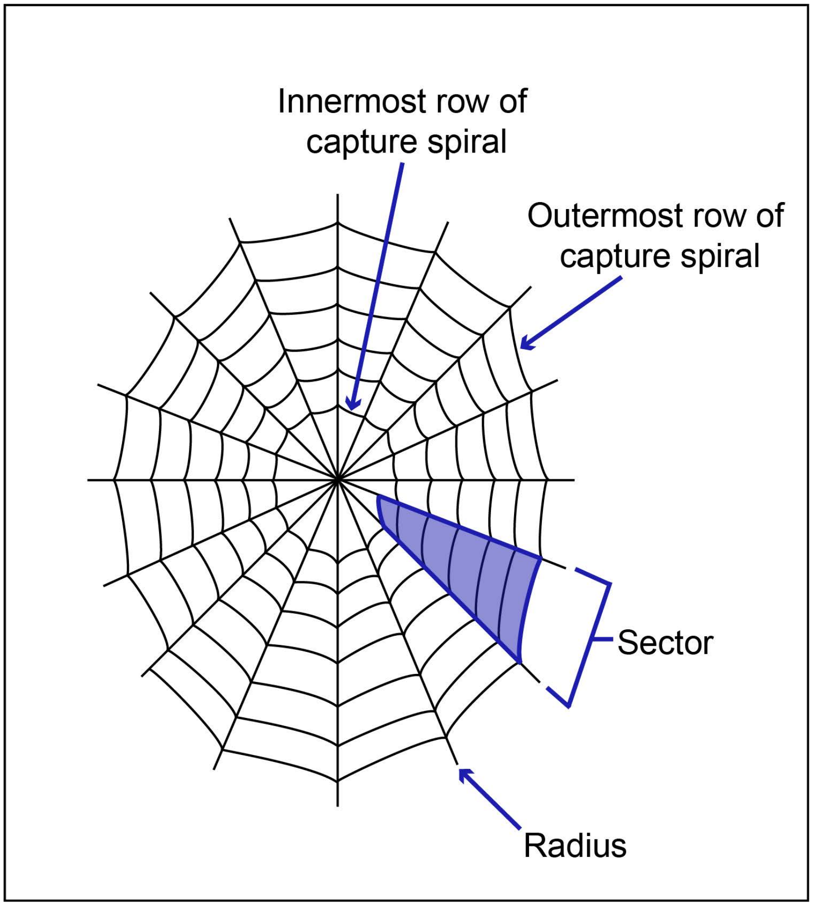

Figure 1: Schematic of a *Tetragnatha* orb web, with key structures (radius, capture spiral, sector) labeled.

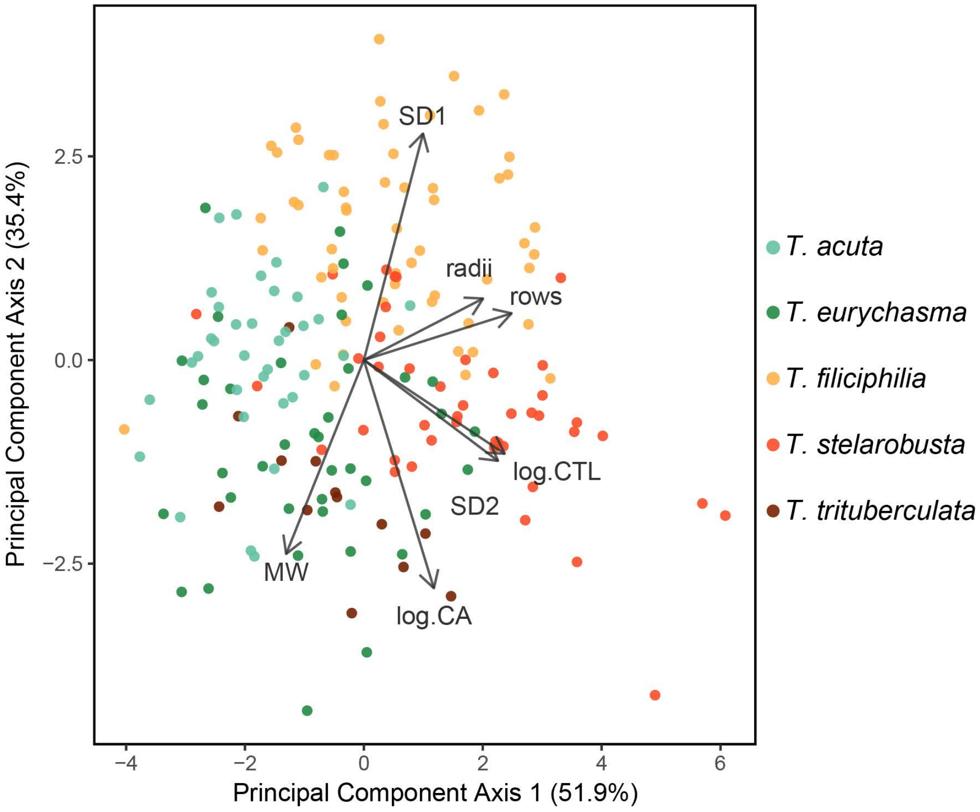

Figure 2: Multivariate analysis of web architecture values across species of *Tetragnatha*. Principal component axes 1 and 2 cumulatively explain 87% of variance. Arrows represent standardized loadings for each web architecture variable included in the analysis.

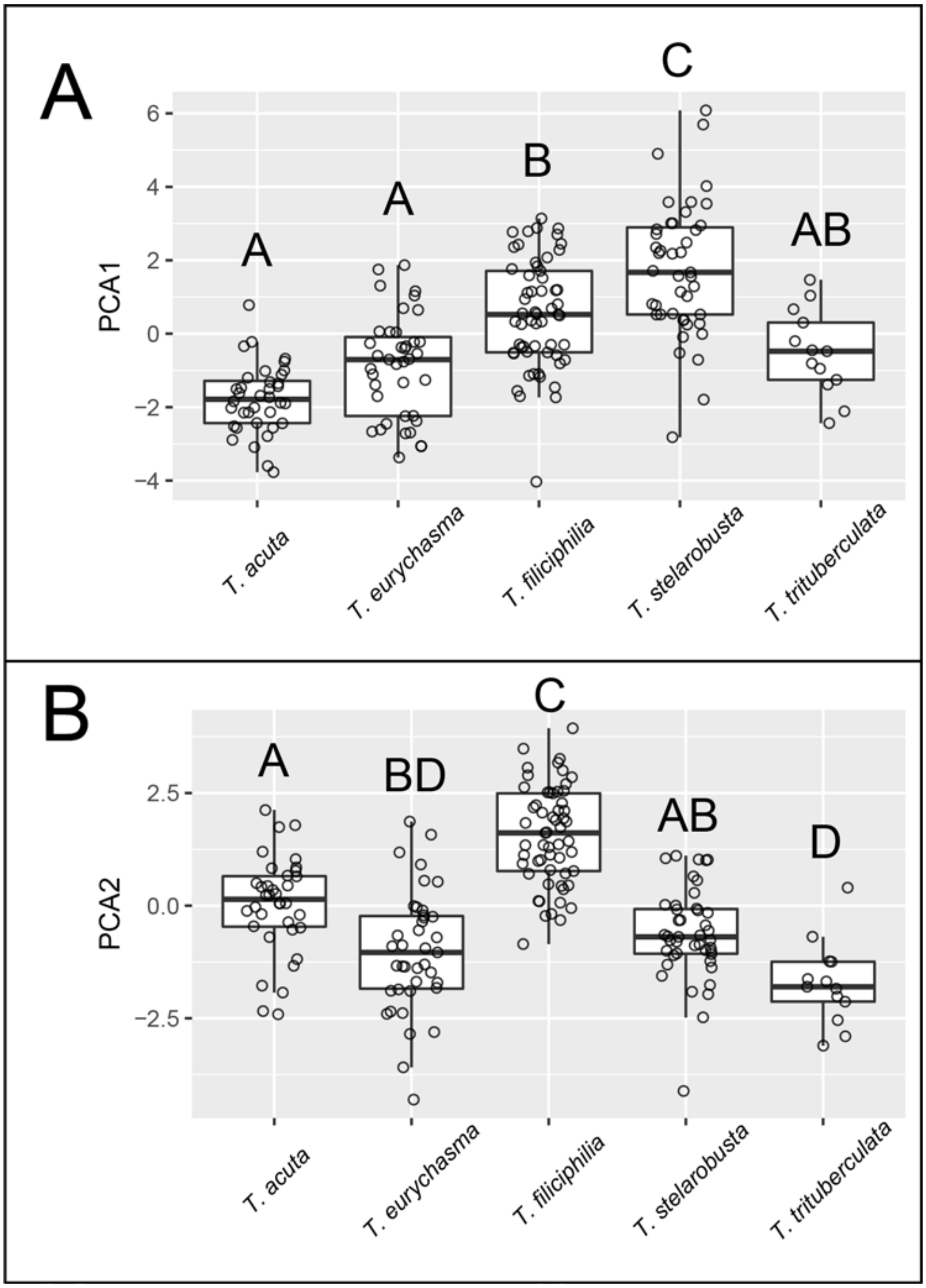

Figure 3: Web architecture PC1 and PC2 values for species of web-building *Tetragnatha*.

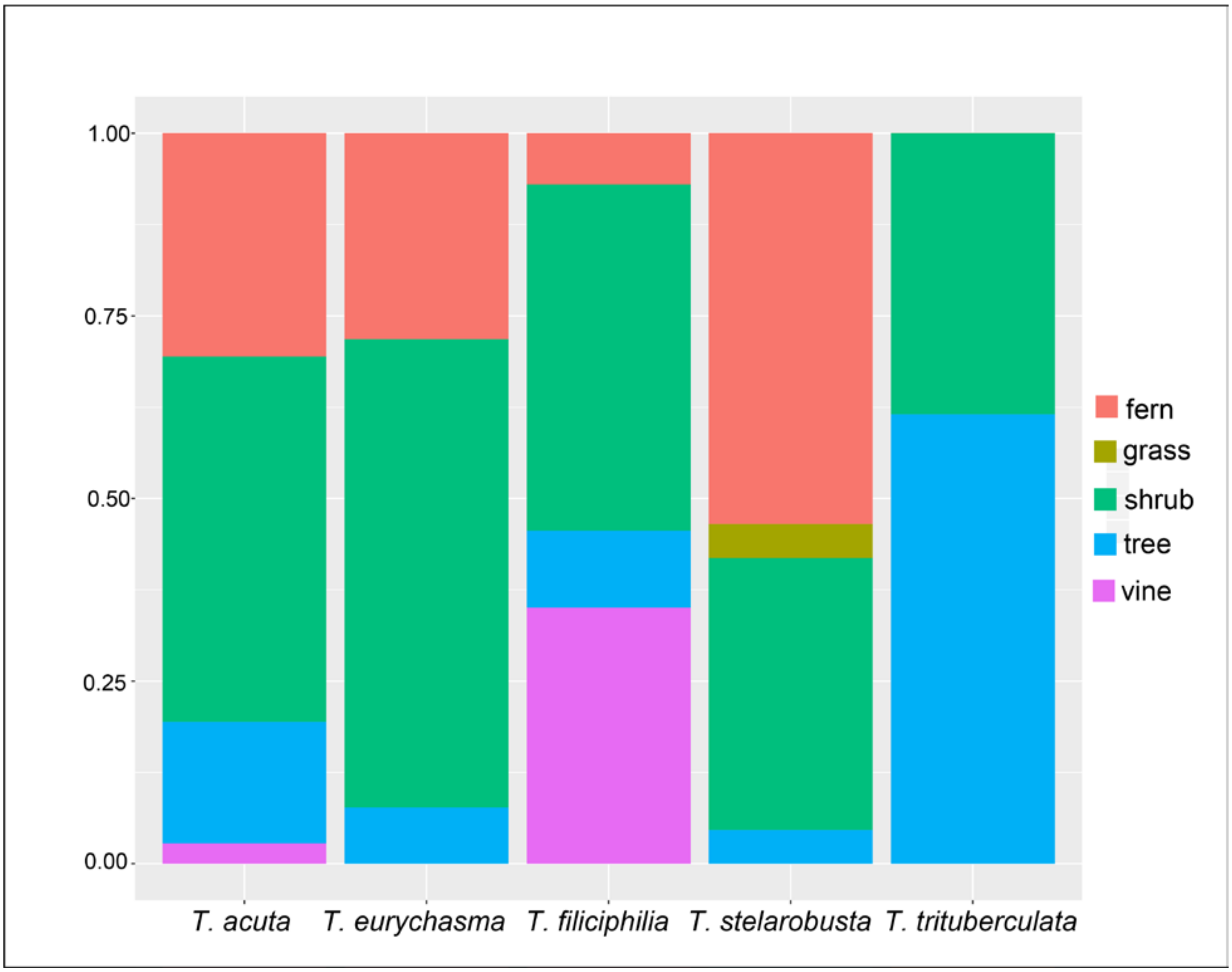

Figure 4: Stacked bar plots showing vegetation-level differences in *Tetragnatha* web placement.

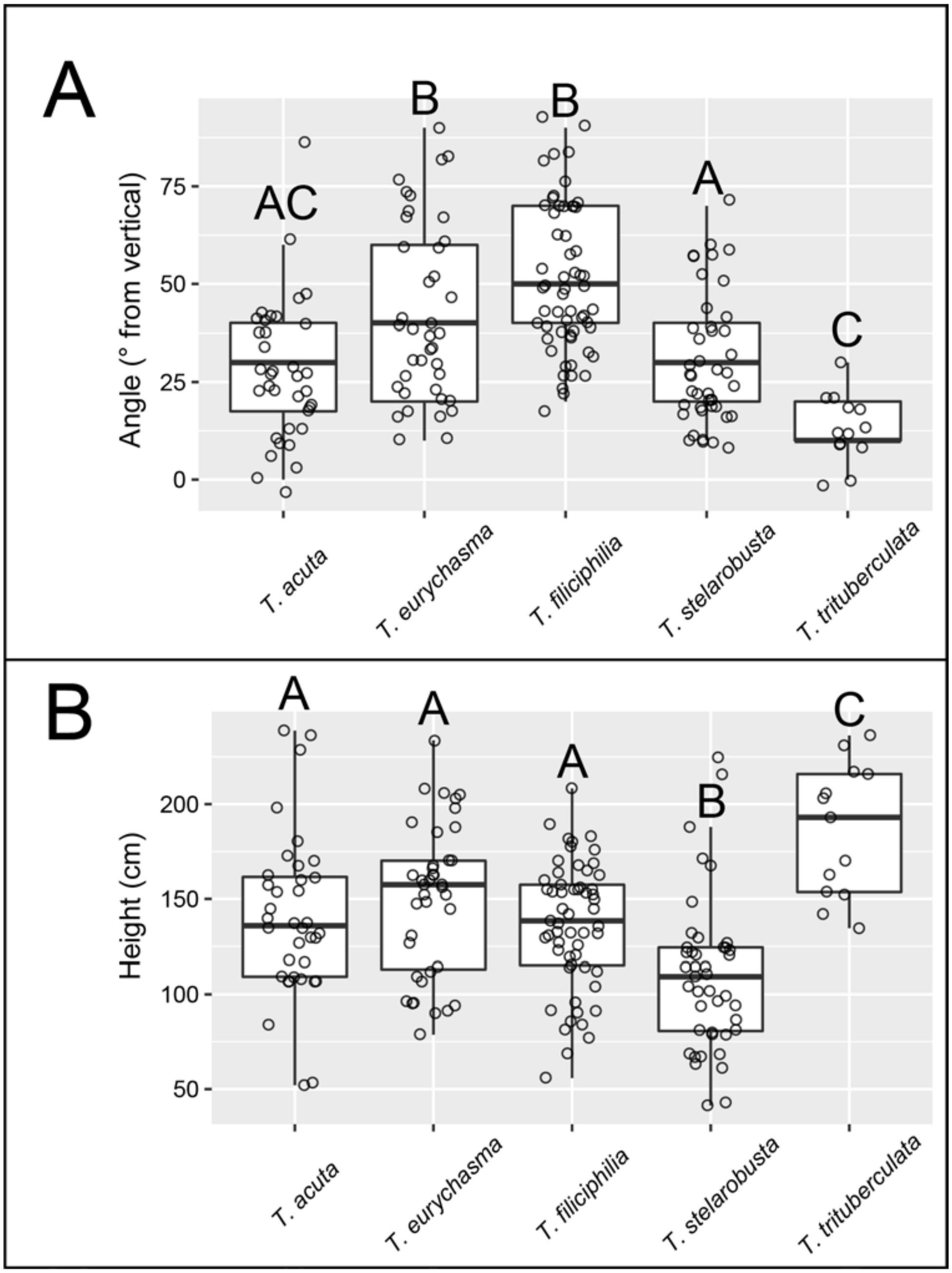

Figure 5: Boxplots showing angle (0° = vertical, 90° = horizontal) and height above ground (cm) of webs for each web-building species.

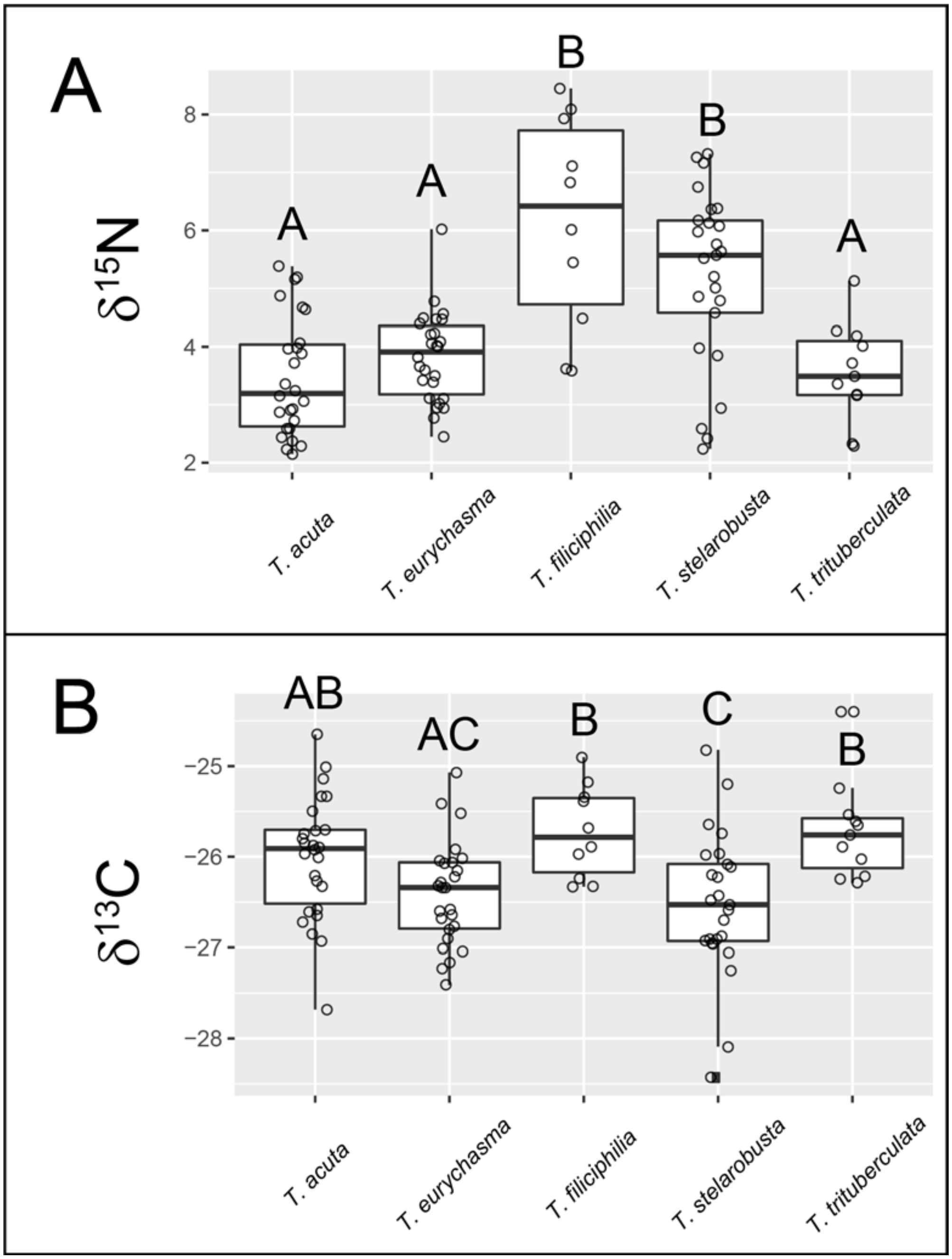

Figure 6: Boxplots of δ^{15}N and δ^{13}C across species of web-building *Tetragnatha*.

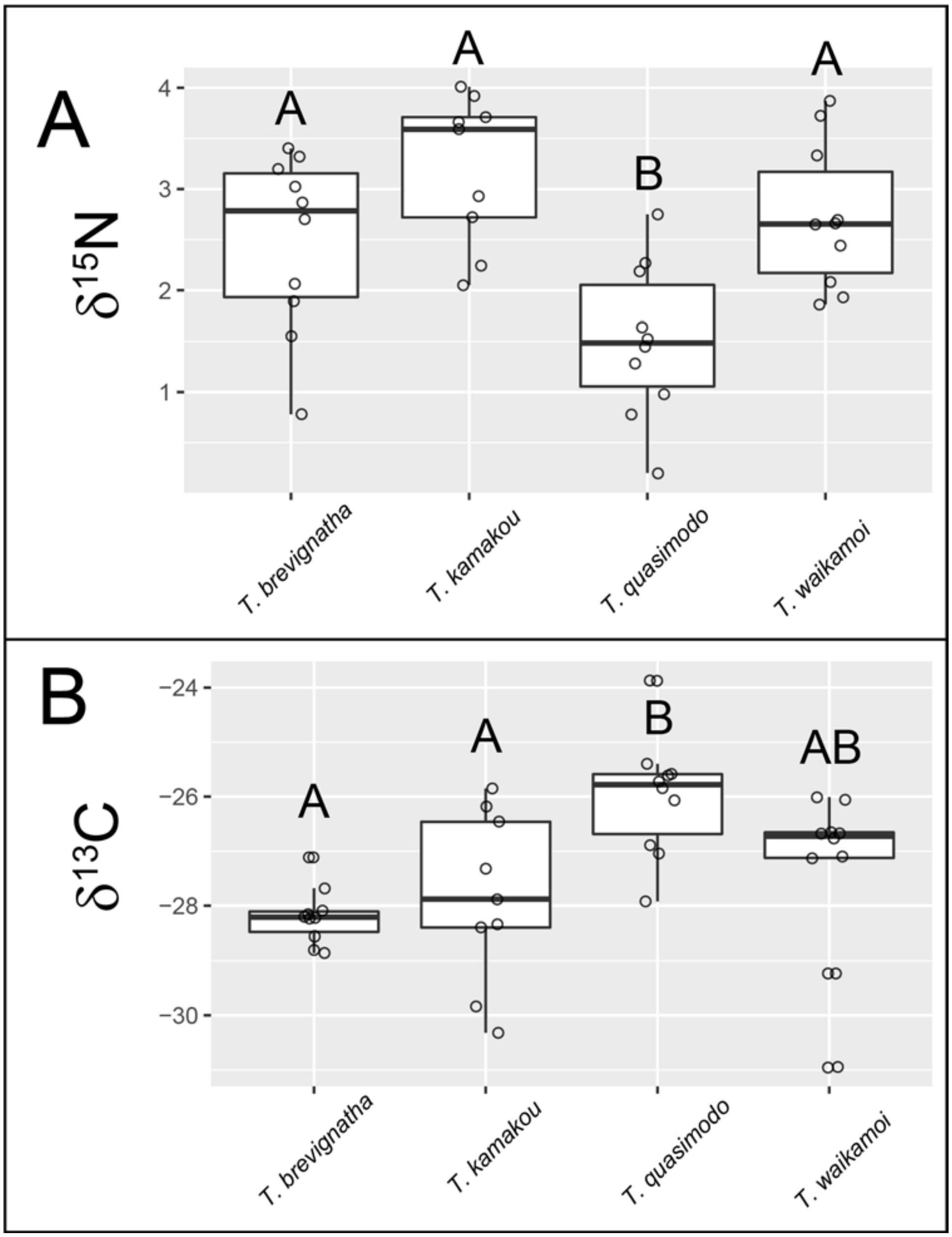

Figure 7: Boxplots of δ^{15}N and δ^{13}C across species of Spiny Leg (actively hunting) *Tetragnatha*.

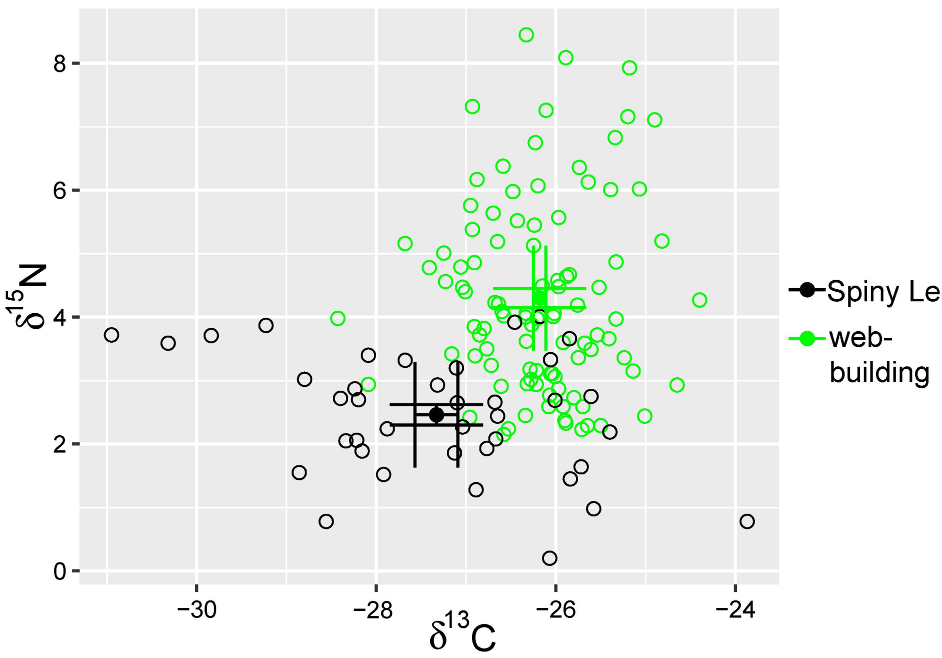

Figure 8: Scatterplot of δ^{15}N and δ^{13}C in web-building versus Spiny Leg *Tetragnatha*.

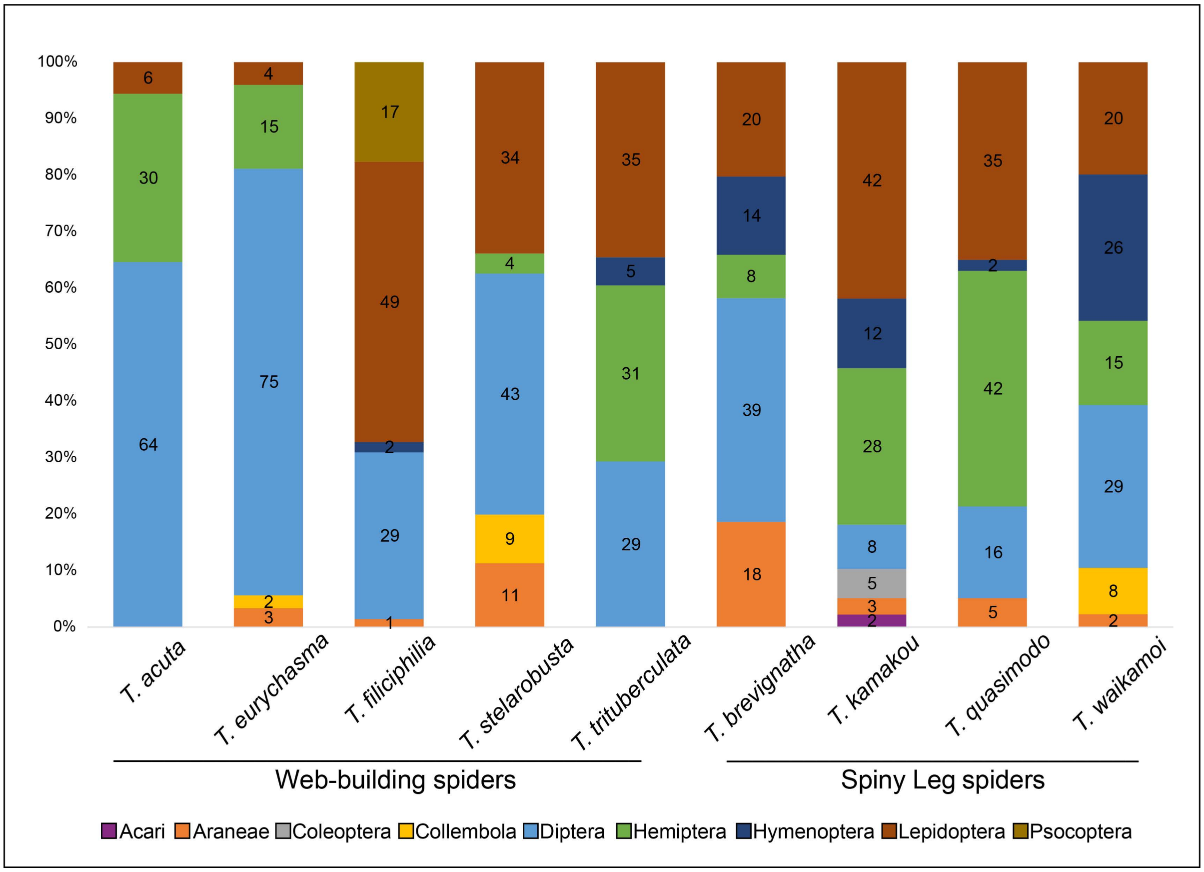

Figure 9: Prey orders detected by molecular gut content analysis in five species of web-building and four species of Spiny Leg *Tetragnatha*: percent composition by spider species.

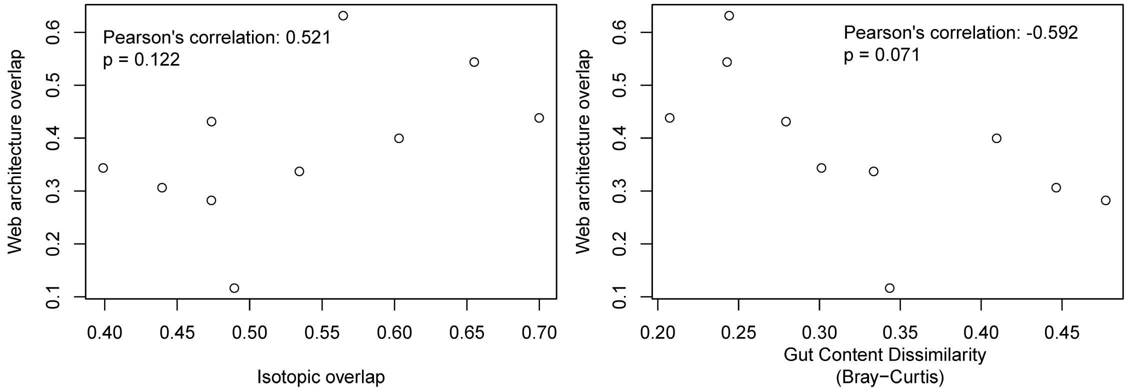

Figure 10: Correlation tests for web hypervolume overlaps with isotopic hypervolume overlaps and gut content beta diversity in web-building *Tetragnatha*.

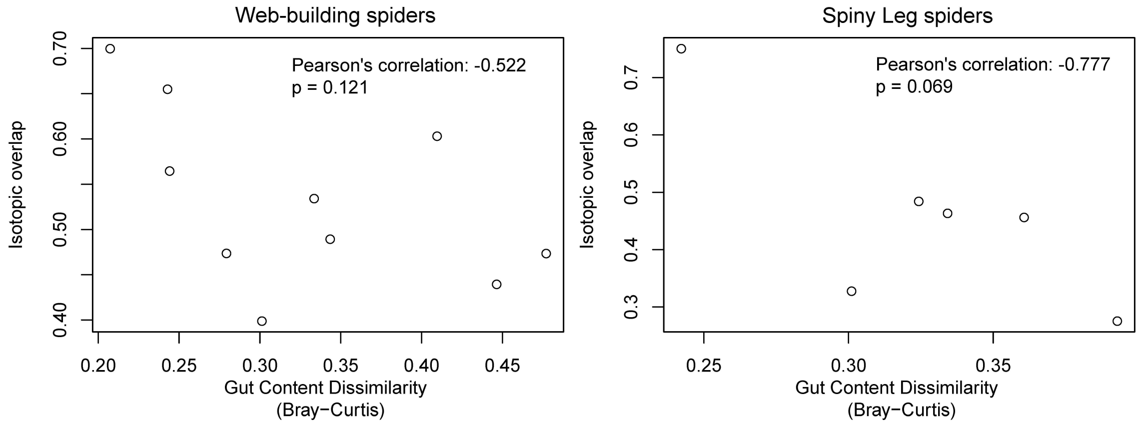

Figure 11: Correlation test for isotopic hypervolume overlaps with gut content beta diversity in web-building and Spiny Leg *Tetragnatha* species.

Chapter 3

A multilocus phylogeny of the adaptive radiation of Hawaiian long-jawed spiders (*Tetragnatha* spp)

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Abstract

Understanding the ecological and evolutionary processes that result in adaptive radiation has long been a goal of biologists. The long-jawed orbweaver spiders (*Tetragnatha* spp) of Hawaii present a rich and promising system for probing these questions. Although much is known about the ecology of these spiders, there has not yet been a comprehensive phylogenetic hypothesis for every known species in the adaptive radiation of Hawaiian *Tetragnatha*. A phylogeny for the entire radiation is needed in order to test the ways in which ecological divergence, convergent evolution, and environmental filtering may have played a role in forming the present-day diversity of these spiders. Here, we present a phylogeny based on data from ten genetic markers and containing representatives of every known species of Hawaiian *Tetragnatha*. We find convincing evidence that the Spiny Leg *Tetragnatha* and the web-building *Tetragnatha* are not reciprocally monophyletic, and therefore likely resulted from separate colonization events. We also find evidence for both ecological divergence within islands and repeated evolution of certain morphological and ecological phenotypes in multiple clades within the radiation. Although these patterns of divergence and convergence have already been demonstrated within the Spiny Leg clade, our study is the first to show that similar phenomena occurred in the web-building clade. Additionally, we find evidence for ecological niche conservatism in some clades. Our results suggest that the adaptive radiation of Hawaiian *Tetragnatha* arose as a result of multiple different evolutionary processes. By assessing the evolutionary relationships among these species, we come one step closer to understanding the ways in which ecology has affected evolution, and vice versa, in this system.

Introduction

Adaptive radiation is defined as the rapid diversification of a single ancestor into multiple lineages with contrasting ecological characteristics (Schluter 2000). Examples of adaptive radiation have been identified in a multitude of systems and at a wide range of taxonomic scales, from members of a single genus in an isolated habitat (e.g. the Hawaiian silverswords, Witter and Carr 1988) to the entire extant fauna of Neoavian birds (Suh *et al*. 2015). Adaptive radiation provides the ideal context in which to examine why and how certain lineages undergo rapid diversification into many descendants, all filling slightly different ecological niches from their ancestor. However, despite centuries' worth of research in diverse systems, predicting which taxa are likely to undergo adaptive radiation and identifying the phenomena that drive this process remains challenging. Here, we use an adaptive radiation in Hawaii to explore the processes that drive evolutionary and ecological diversification in an age-structured island system.

Adaptive radiation can arise under a number of different conditions, and is not necessarily confined to island or island-like systems (Stroud and Losos 2016); however, the chief prerequisite is the availability of open niche space, i.e. ecological opportunity (Erwin *et al*. 1987, McCune 1997, Gavrilets 2005, Stroud and Losos 2016). This ecological opportunity may, for example, come in the form of a newly available habitat (Grant and Grant 2011, Seehausen 2006, Losos 2011) or the local extinction of potential competitors or enemies (Friedman 2010, Chen and

Benton 2012). The radiation process begins when the ancestral lineage first occupies the new niche space, e.g. by establishing itself on a new island. If the lineage possesses enough genetic and/or behavioral variability, it will be able to expand into new niches that have not yet been filled by potential competitors. As the lineage diversifies and new species accumulate, these species may begin to compete for resources, and may consequently be subjected to selection for niche partitioning – i.e., for exploiting resources that are potentially more abundant or available than resources already being exploited by congeners in the habitat. This eventually drives ecological divergence among genetically diverging, sympatric species, and the same process may be repeated several times as the lineage disperses to new habitats. Under this model, priority effects – specifically, niche preemption – may play a critical role in determining which lineages are likely to undergo adaptive radiation (Fukami 2015, Tan *et al.* 2017). In its simplest terms, this means that the first arrivals that possess sufficient genetic variation will be more likely to radiate than later colonists, which will arrive after much of the niche space is already occupied. However, some have argued that environmental constraints may be more important than priority effects in determining the evolutionary trajectory of a lineage, and there is growing evidence that habitat filtering – the inability for species to become established in habitats that do not meet their ecological needs – may be more important than priority effects in some systems (Medeiros *et al*. 2015, Pontarp *et al*. 2012, Savage and Cavender-Bares 2012).

The long-jawed orb-weaving spiders (genus *Tetragnatha*) of Hawaii comprise a speciose and ecologically diverse adaptive radiation (Gillespie 2015). While the genus has a global distribution, most of its members share a stereotyped morphology and ecological niche, with elongate bodies and legs, long chelicerae (jaws), and a tendency to build orb webs near or over bodies of water (Kaston 1978). Within Hawaii, by contrast, *Tetragnatha* has diversified into an array of morphologies and ecological affinities that are strikingly different from the largely homogeneous characteristics of the genus in the rest of its range. The Hawaiian radiation consists of two major clades: one of web-builders, which use orb webs to capture their prey (the ancestral condition; Kaston 1978), and another, known as the Spiny Leg Clade (Gillespie 1991, 2002b), that has lost the web-building trait and instead hunts actively.

With 31 described species and an estimated 10-15 additional undescribed morphospecies as well as strong evidence for both divergent evolution within islands and convergent evolution of "morphotypes" in distinct habitats (Blackledge and Gillespie 2004, Gillespie 2004), the Hawaiian radiation of *Tetragnatha* presents an ideal system for the study of evolution in an ecological context. Here, we present a phylogenetic hypothesis for the entire Hawaiian *Tetragnatha* radiation across its range on the archipelago. Having a taxonomically comprehensive, well-resolved phylogenetic hypothesis for the whole radiation makes it possible to explore the extent to which different factors have driven the genetic and ecological diversification that this lineage has undergone: for example, whether lineages have diverged within habitats, phenotypes have evolved convergently in different clades, and/or ecological constraints have shaped the present-day distributions of lineages.

We first compare our recovered ML and Bayesian phylogenies with previous work (Gillespie 1999, 2004, 2005; Blackledge and Gillespie 2004), which presented phylogenetic hypotheses for smaller subsets of Hawaiian *Tetragnatha* species. We then address deep evolutionary relationships within the adaptive radiation, using our results to explore the putative number of colonization events from which the Hawaiian radiation arose. Finally, using the phylogenetic framework we obtain with our analysis, we test the following two non-exclusive hypotheses to explain the present-day diversity of Hawaiian *Tetragnatha*: 1) ecological opportunity was a primary driver of speciation, resulting in strong ecological divergence within clades such that closely related species found in sympatry are more ecologically different from one another than they are from more distantly related and/or allopatric species; and 2) habitat filtering played an important role in speciation, such that ancestral lineages were only able to establish themselves in habitats that met their ecological needs; and subsequently, due to Hawaii's high geological complexity and the isolation of such habitats from one another, the loss of gene flow ultimately led to the formation of new species. If (1) is supported, then we expect to see ecologically divergent species grouping together into clades. We also expect convergent evolution of ecological/morphological traits on multiple islands because similar dynamics of niche availability over time – i.e. open niche space initially, followed by a gradual filling of niches as species accumulate – are expected to have occurred repeatedly on the different islands. If (2) is supported, then we expect to see phylogenetic clustering of ecologically similar species, e.g. a group of high-elevation species may form a clade even if they occur on different islands. By providing a phylogenetic hypothesis for the whole radiation of Hawaiian *Tetragnatha*, we will create the framework necessary for testing these predictions as well as revealing new information on the evolutionary and ecological dynamics underlying this adaptive radiation.

Methods

Specimens used

The dataset used in this phylogeny was assembled with the aim to represent every major population of every *Tetragnatha* species endemic to Hawaii. Because there are approximately 50 known species of Hawaiian *Tetragnatha*, some occurring on more than one island, the specimens used here were aggregated from multiple collecting trips taken to Hawaii between 1992 and 2017. All spiders collected on these trips were hand-captured into individual vials and stored in 95% ethanol at -20° C in Prof. Rosemary Gillespie's lab at UC Berkeley. In the course of extensive work on the Hawaiian *Tetragnatha*, Gillespie (1991, 1992, 1994, 2002b, 2003, unpubl. data) has obtained detailed information on the ranges occupied by the species in this radiation. Based on this information, appropriate localities and islands for each species (and in some cases, each population) were determined, and specimens from these localities were chosen accordingly. However, for some populations, no specimen was available – i.e., although specimens had been collected previously, their DNA had already been consumed for earlier studies. In these cases, older sequence data taken from earlier studies (Gillespie *et al*. 1994, 1997; Gillespie 1999, 2005) were incorporated into the final dataset. The final dataset was composed of 87 "new" specimens, from which DNA was freshly extracted, and 46 individuals represented by older sequence data

(Gillespie *et al*. 1994, 1997; Gillespie 1999, 2005)(Suppl. Table 1). Sampling localities for each species are shown in Fig. 1. Note that, when referring to islands, we use “Hawaii” synonymously with “Big Island” (the youngest and southeastern-most island in the archipelago). We also refer to Maui Nui (Macdonald and Abbott 1970), which is the complex of islands that includes Maui, Molokai, Lanai and Kaho’olawe. Many *Tetragnatha* species are endemic to the Maui Nui complex and were therefore sampled from more than one island within this complex.

Molecular lab work

DNA was extracted from leg tissue of 87 specimens using the Qiagen PureGene extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Leg tissue was macerated, after which Qiagen Cell Lysis Solution and Proteinase K were added to each sample. Tissue was lysed for six hours at 55° C, followed by the addition of RNase A and a 30-minute incubation at 37° C to remove RNA. Protein precipitation and DNA pelleting were then performed according to the Qiagen PureGene protocol. DNA pellets were hydrated with 30 uL of Qiagen DNA Hydration Solution, after which they were visualized on a 1.5% agarose gel to confirm DNA quality. Extracts were stored at 4° C until they were needed for PCR.

PCR was used to amplify a total of ten markers (summarized in Table 1), of which two were protein-coding mitochondrial markers (Cytochrome Oxidase I (COI) and Cytochrome B (CytB)), two protein-coding nuclear markers (Actin and Histone H3 (H3)), two non-coding ribosomal mitochondrial markers (12S and 16S), and four non-coding ribosomal nuclear markers (18SM, 28SM, ITS2 and SSU). These markers were selected based on Krehenwinkel *et al*. (2018a), in which they were established as effective phylogenetic markers for spiders (including *Tetragnatha* spp). PCR protocols for all markers except Actin followed the protocols described in Krehenwinkel *et al*. (2018a), with one multiplex for mitochondrial markers 16S, COI and CytB; one multiplex for nuclear markers 18SM, SSU, 28SM and H3; and separate PCR reactions for 12S and ITS2. For Actin, we followed the same thermocycler protocol as Krehenwinkel *et al*. (2018a) but used an annealing temperature of 47° C. We used custom-made primers containing a 30-bp TruSeq tail on the 5’ end of the forward primer and a 24-bp TruSeq tail on the 5’ end of the reverse primer. Each of these tails binds to a complementary sequence on a TruSeq indexing primer in a second round of PCR (described below). PCR products were visualized on a 1.5% Agarose gel with a 100-bp ladder (New England BioLabs, Ipswich, MA, USA) for fragment size assessment, then cleaned of leftover primers with 1X AMPure beads XP (Beckman Coulter, Indianapolis, IN, USA).

Because we performed DNA sequencing on an Illumina MiSeq platform, the PCR products were run through a second round of PCR for indexing. In this reaction, the PCR products were bound to forward and reverse Illumina TruSeq indexing primers containing 1) a sequence complementary to the TruSeq tails included in the primers from the first round of PCR; 2) an 8-bp indexing barcode; 3) a sequencing tail designed to bind to the sequencing primers in the MiSeq flow cell. The design of these indexing primers was based on Lange *et al*. (2014). For each PCR sample, a unique combination of forward and reverse barcoding primers was used so that the sample could later be identified from the sequencing run. Indexed PCR products were visualized on a 1.5% agarose gel, cleaned of residual primers with 1X AMPure beads, then pooled together at roughly equal amounts (i.e. ng of DNA) based on gel band strength. The pool was quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and diluted to 4 nM concentration. The 4-nM sample was run on an Illumina MiSeq with V3 chemistry (600 cycles) and 2 x 300 bp reads at the California Academy of Sciences' Center for Comparative Genomics (San Francisco, CA, USA).

Sequence processing

Sequences were demultiplexed by index barcode combination, and forward and reverse reads were assembled with Paired-End reAd mergeR (PEAR; Zhang *et al*. 2014). Assembled reads were then quality-filtered and converted to FASTA format with FastX-Toolkit (Gordon and Hannon 2010). FASTA files were dereplicated and then clustered into operational taxonomic units (OTUs) at 97% similarity with USEARCH (Edgar 2010), with a *de novo* chimera removal step included in the OTU clustering.

Because of sequencing error, heterozygosity, coamplification of parasites such as fungi or nematodes, and NUMTs (mitochondrial sequences transposed into the nuclear genome), some specimens had more than one OTU for a given marker. OTU-clustered FASTA files were therefore checked by hand in MEGA7 (Kumar *et al*. 2016). Sequences were first checked against the NCBI nucleotide database using BLASTn (Altschul *et al*. 1990) to filter out non-spider hits. NUMTs were then identified in protein-coding mitochondrial markers (COI and Cytochrome B) by translating the sequences and finding OTU sequences that were interrupted by a stop codon. After these initial filtering steps, it was generally easy to identify the correct OTU as the one with the largest number of reads. However, when OTU size was not sufficient to distinguish correct from incorrect OTUs, a neighbor-joining tree was built using all specimens. From these trees, erroneous sequences could be easily recognized because they fell outside of clades formed by conspecific specimens.

Once a single correct sequence had been identified for each marker from each specimen, sequences were aligned in MEGA7 using ClustalW (Thompson *et al*. 1994) with a gap opening penalty of 15 and a gap extension penalty of 6.66 for both pairwise and multiple alignments, and a 0.5 transition weight. Gap opening and extension penalties determine the frequency and length of gaps that are permitted in the alignment, with higher values corresponding to fewer and shorter gaps. Transition weight is assigned such that a weight of 0 causes all transitions (A ⟷ G and C ⟷ T) to be treated as mismatches, while a weight of 1 causes transitions to be treated as matches. The values we selected (15 for gap opening and 6.66 for gap extension; 0.5 for transition weight) are the defaults in MEGA7, and produced reasonably accurate alignments, which were then further checked by hand. In ITS2, which contains regions of very high uncertainty, the GBlocks software (Castresana 2000) was used to identify and remove these regions.

The 46 "old" sequences used in the analysis were all either 12S, 16S or COI taken from earlier studies (Gillespie *et al*. 1994, 1997; Gillespie 1999, 2005). These sequences were pasted into the

FASTA files for the "new" alignments of these markers, then aligned as described above. After this, all ten markers (12S, 16S, 18SM, 28SM, Actin, COI, CytB, H3, ITS2 and SSU) were concatenated with a custom-made Python script.

Phylogenetic analysis

A final concatenated alignment of 3326 bp (see Table 1 for lengths of individual markers) and 133 samples was used in the phylogenetic analysis. The FASTA file was converted to Phylip and Nexus formats in Geneious 11.1.5 (https://www.geneious.com) and run in PartitionFinder 2.1.1 (Lanfear *et al*. 2016) on the CIPRES Science Gateway (Miller *et al*. 2010) to determine the best partitioning scheme (with lowest AICc value) and appropriate models for each partitioned subset (see Table 2). This best partitioning scheme was used for both Maximum Likelihood and Bayesian phylogenetic analyses.

A Maximum Likelihood (ML) phylogeny was generated using IQTree (Nguyen *et al*. 2015) on the IQTree server with ultrafast bootstrapping (Minh *et al*. 2013), using a GTR+GAMMA substitution model. A Bayesian tree was then generated in MrBayes 3.2.6 (Ronquist *et al*. 2012) on the CIPRES Science Gateway with 10 million generations and substitution models for each partition as summarized in Table 2. Trees were rooted with the clade formed by North American species *Tetragnatha versicolor* and *T. viridis*, and were formatted into figures using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator CC 22.0.1 (Adobe Systems, San Jose, CA, USA).

Results

Tree topologies: summary

Tree topology is largely congruent between the ML (Fig. 2) and Bayesian (Fig. 3) analyses, both of which recover a well-supported Spiny Leg clade and a separate clade of web-building species.

In the Spiny Leg clade, the three species from Kauai (*T. pilosa*, *T. mohihi* and *T. kauaiensis*) form a clade basal to the rest of the Spiny Leg species. Within the the remaining Spiny Leg species, there are two major clades: one containing *T. quasimodo* as sister to the "Small Brown Spiny Legs" (*T. kukuiki*, *T. kikokiko*, *T. kukuhaa*, *T. obscura* and *T. anuenue*), with three Oahu species (*T. perreirai*, *T. polychromata* and *T. tantalus*) forming a clade at the base of this sister pair; and the other containing the "Eastern Green" clade with *T. kamakou* from Maui as their sister, and a sister clade composed of *T. kamakou* from Molokai and *T. restricta* from Maui and the Big Island. *T. kamakou* is thus polyphyletic, with the Maui population falling as sister to the "Eastern Greens" and the Molokai population grouping with *T. restricta.* The Big Island population of *T. restricta* is paraphyletic with respect to the Maui population. Additionally, *T. obscura* (in the "Small Brown Spiny Leg" clade) is paraphyletic with respect to *T. kukuhaa*.

Within the web-builders, the North American species *T. pallescens* falls as the closest outgroup. Next are *T. hawaiensis* and the Kauai morphospecies known as *T.* sp. "Waves," which group together as sisters (97% bootstrap support in ML analysis, though just 55% posterior probability in Bayesian analysis). Within the radiation of web-builders (i.e. internal to the node where *T. hawaiensis* and *T.* sp. "Waves" split off), the morphospecies *T.* sp. "Emerald Ovoid" and *T.* sp. "Bicolored Jaws," both from Oahu, fall most basal and as sister species. *T. paludicola* from East Maui is next-most basal. For the remaining species, ML analysis recovers two large clades with moderate (71-79%) bootstrap support, whereas Bayesian analysis finds a polytomy of four smaller clades. In the Bayesian tree, these clades are: 1) a tritomy of *T. paludicola* from Molokai, the "Eastern Forest" clade, and the "Oahu Elongate Forest" clade of *T. lena*, *T. limu* and *T. palikea*; 2) a group in which the Big Island *T. acuta*/*T. kea* fall as sister to a clade of Oahu *T.* sp. "Eurylike" with Oahu *T.* sp. "Pointed Abdomen," with Maui *T. acuta*/*T. albida* falling basal to this sister pair (in other words, "*T. acuta*" is paraphyletic with respect to Oahu *T.* sp. "Eurylike" and *T.* sp. "Pointed Abdomen"); 3) the "Fern Dweller" clade of *T. filiciphilia*, *T.* sp. "Red Star," and *T.* sp. "Golden Dome"; and 4) a tritomy of West Maui *T. paludicola*, *T. maka*, and the "Mountain Top Clade" of dry forest specialists with the *T.* sp. "Long-Clawed Legs" morphospecies. In the Maximum Likelihood analysis, relationships that appeared as polytomies in Bayesian analysis are resolved at moderate to high support (>50% bootstrap for all nodes, and most nodes 70-100% bootstrap support). The "Oahu Elongate Forest" and "Eastern Forest" clades appear to be sisters (66% bootstrap support). The "Mountain Top" clade/*T. maka*/West Maui *T. paludicola* appears to be sister to the clade of "*T. acuta*"/ *T.* sp. "Eurylike" (73% bootstrap support), and the clade formed by this whole group is sister to the "Fern Dweller" clade (71% bootstrap support). *T. paludicola* is polyphyletic, with each of its three major populations appearing in a completely different place within the web-building clade: the East Maui population is basal to most of the radiation (100% bootstrap support, 99% posterior probability); the Molokai population groups with the "Oahu Elongate Forest" and "Eastern Forest" clades (79% bootstrap support, 97% posterior probability); and the West Maui population groups with the "Mountain Top" clade and *T. maka* (93% bootstrap support, 89% posterior probability).

Deeper evolutionary relationships within the radiation

The Spiny Leg clade is recovered as sister to a larger clade of web-building species, which includes the mainland outgroup *T. pallescens*. This shows that web-building Hawaiian *Tetragnatha* are more closely related to a mainland species than to their fellow Hawaiian species in the Spiny Leg clade. However, no mainland sister to the Spiny Leg clade was recovered in this analysis; our two other mainland species, *T. versicolor* and *T. viridis*, fall basal to all other taxa analyzed here. The long branch length of *T. hawaiensis* fits with the findings of earlier studies (Gillespie *et al*. 1994, 1997; Casquet *et al*. 2015), which strongly suggest that this species originated from a colonization event separate from the rest of the Hawaiian radiation. Additionally, the position of *T.* sp. "Waves" as putative sister to *T. hawaiensis* indicates that this lineage also falls outside of the main web-building radiation, either having shared a recent common ancestor with *T. hawaiensis* or having originated from still another colonization event.

Ecological divergence: within-island diversification

Both ML and Bayesian topologies indicate several instances of putative ecological divergence within habitats, i.e. monophyly of ecologically distinct species within single islands. This pattern is found in Spiny Leg spiders of Kauai (in which all three Kauai species, each belonging to a different "ecotype" (Gillespie 2004), form a clade together) and Oahu (excluding *T. quasimodo*); in web-builders, the clade of *T.* sp. "Emerald Ovoid" and *T.* sp. "Bicolored Jaws" on Oahu, and the "Eastern Forest" clade (excluding *T. perkinsi*, which appears to have split off from the common ancestor to the three Maui Nui species). Conversely, the web-building "Oahu Elongate Forest" clade appears to have given rise to three different species, all confined to different habitats on Oahu, but these species are morphologically and ecologically similar to one another (Gillespie 2003) and thus show no evidence of having undergone ecological divergence.

Morphological and ecological convergence

Repeated evolution of Spiny Leg "ecotypes" (Gillespie 2004) is recovered in our analyses. The "large brown" ecotype occurs twice independently, in the form of *T. pilosa* (Kauai) and its relatively distant relative *T. quasimodo* (all other islands). The "small brown" ecotype is found in the independent lineages of *T. mohihi* (Kauai), *T. restricta* (Maui Nui and Big Island), and the "Small Brown Spiny Leg" clade of *T. kukuiki* (Oahu), *T. kikokiko* (Maui), and *T. anuenue*, *T. kukuhaa* and *T. obscura* (Big Island). The "green" ecotype also appears in three different places on the tree: on Kauai with *T. kauaiensis*, on Oahu with *T. polychromata* and *T. tantalus*, and on Maui Nui and Big Island with the "Eastern Green" clade of *T. brevignatha*, *T. macracantha* and *T. waikamoi*. Finally, the "maroon" ecotype occurs separately three times: at the base of the Oahu clade as *T. perreirai*, at the base of the "Eastern Green" clade as Maui *T. kamakou*, and as sister to *T. restricta* as Molokai *T. kamakou*.

Patterns in web-builders are less clear because formal "ecotypes" have not been established in the literature. However, Gillespie (2002a, unpubl. data) has recognized certain recurring morphological phenotypes within the web-builders: "Elongate Forest," characterized by brownish coloration and a cigar-shaped abdomen most reminiscent of the typical *Tetragnatha* body shape in mainland species; "Eurylike," smallish spiders possessing an oval abdomen with a lateral black wave-shaped color pattern; and "Pointed Abdomen," also relatively small in size, with an abdomen that is roughly triangular in side view and raised to a central, pointed hump. We find the "Elongate Forest" phenotype in Oahu, with *T. lena*, *T. limu* and *T. palikea* (and also *T. uluhe*, a dry forest specialist which falls within the "Mountain Top" clade); in Maui Nui with *T. stelarobusta*; and in dry forests across the archipelago, with the undescribed species in the "Mountain Top" clade. The "Eurylike" morphology occurs in three places on the phylogeny: first, as the distantly related Kauai *T.* sp. "Waves," next as Oahu *T.* sp. "Eurylike," and lastly as *T. eurychasma*, of Maui Nui. Finally, the "Pointed Abdomen" morphology occurs in two places: first, in the form of *T. maka*, which appears to be closest to the "Mountain Top" clade and West Maui *T. paludicola*; and second, with the clade of *T. acuta, T. albida*, *T. kea*, Oahu *T.* sp. "Pointed

Abdomen" and Oahu *T.* sp. "Eurylike" (which is not of the putative "Pointed Abdomen" morphotype, but is apparently nested within this group).

Habitat filtering

One clade shows strong evidence for habitat filtering: the dry forest specialists within the "Mountain Top" clade. This group, composed of several "Elongate Forest" morphospecies from Kauai, Maui Nui and Big Island as well as *T. uluhe* from Oahu, is found only in dry forest habitats on the different islands. This clade is most closely related to *T.* sp. "Long-Clawed Legs," a relatively rare elongate species with unusually long claws on its tarsi (distal-most leg segments). Additionally, the newly designated "Fern Dweller" clade of *T. filiciphilia*, *T.* sp. "Red Star" and *T.* sp. "Golden Dome" may be an example of niche conservatism and possible habitat filtering. These three species appear to have a strong affinity for ferns as web-building substrates; indeed, *T. filiciphilia* is so named because of its association with ferns (Gillespie 1992). Although published data on the undescribed species *T.* sp. "Golden Dome" and *T.* sp. "Red Star" are lacking, both of these species are found with overwhelming frequency on the undersides of *Cibotium* spp. tree fern fronds (pers. obs.).

Discussion

Building on our understanding of evolutionary relationships within the radiation

The phylogeny presented here expands upon previously published phylogenetic analyses of *Tetragnatha* which examined relationships within subsets of this radiation. Many of these earlier studies also relied on molecular data, though with fewer markers than were used in the current study. In general, our trees are congruent with these earlier efforts. This is particularly apparent in the Spiny Leg clade. Using 12S, 16S, COI and allozymes, Gillespie (2004) found relationships among species in this group that are nearly identical to those recovered here: namely, a Kauai and an Oahu clade; monophyly of *T. quasimodo* across its multi-island range; and a clade of "small brown" species that is sister to *T. quasimodo*. Even COI alone recovered results similar to ours for the relationships among *T. brevignatha*, *T. macracantha*, *T. waikamoi*, *T. kamakou* and *T. restricta* (Gillespie 2005), the only difference being the placement of *T. kamakou* from East Maui. Some of our results for web-builders also match earlier work, primarily the recovery of an "Oahu Elongate Forest" clade and a "Mountain Top" clade (Gillespie 1999), and the basal position of morphospecies *T.* sp. "Emerald Ovoid," as well as recovering a clade of Maui forest species (Blackledge and Gillespie 2004). However, by adding several web-building species that were absent from previous phylogenetic analyses, we are able to recover higher resolution on the relationships within this lineage – specifically, we can now identify many putative pairs of sister species, some of which are very well supported. In the course of the current study, we even discovered a new morphospecies, nicknamed *T.* sp. "Red Star," from Hawaii Island, which appears to be sister to *T. filiciphilia*.

Multiple origins of Hawaiian *Tetragnatha*

Previous work (Gillespie *et al*. 1994, 1997; Casquet *et al*. 2015) has shown strong support for the hypothesis that *T. hawaiensis* falls outside of the Hawaiian adaptive radiation, and that it likely originated from a separate colonization event. Unlike the other members of the Hawaiian *Tetragnatha*, *T. hawaiensis* is not confined to one or two islands, but has populations on every island in the archipelago. Moreover, our tree topology suggests that the populations of *T. hawaiensis* are not monophyletic by island but, instead, are mixed among the islands. The position of *T. hawaiensis* and its long branch length also support its status as a separate lineage. Hence, our results fit expectations for Hawaiian *Tetragnatha* having originated from a minimum of two colonization events, *T. hawaiensis* having arrived separately from the rest of the radiation.

What has historically been less certain is whether the two major clades of *Tetragnatha* are monophyletic, or whether the web-builders and Spiny Leg clade came from two separate colonization events. Our results strongly suggest that the latter is true. While some previous phylogenies found *T. hawaiensis* to fall outside of the whole radiation, including the Spiny Leg clade (Gillespie *et al*. 1997, Casquet *et al*. 2015), our results indicate that *T. hawaiensis* is more closely related to the web-builders than to the Spiny Leg clade. Two recent phylogenies found the same pattern, namely that *T. hawaiensis* groups with the web-builders (Krehenwinkel *et al*. 2018a, 2018b). This precludes the possibility that the web-builders could form a clade with the Spiny Leg species, given that *T. hawaiensis* is from a separate lineage. Stronger evidence still for the separate origins of web-builders and the Spiny Leg clade is the position of *T. pallescens*, a North American outgroup, as sister to the clade containing Kauai *T.* sp. "Waves," *T. hawaiensis*, and the rest of the web-builders. It is unfortunate that we have not yet been able to identify a sister outgroup to the Spiny Leg clade; inclusion of a broader taxonomic and geographic range of *Tetragnatha* outgroups could greatly benefit future phylogenetic studies of this group.

Localized niche divergence versus dispersal with niche conservatism

Our results show a fascinating juxtaposition of contrasting evolutionary patterns. One phenomenon we see is within-island diversification: i.e., monophyletic groups of species that occur on the same island, yet differ markedly from each other in their morphology and ecology. The clade of Oahu morphospecies *T.* sp. "Emerald Ovoid" and *T.* sp. "Bicolored Jaws" is a striking example: these two species are greatly different in appearance as well as, to our knowledge, ecological niche. In fact, *T.* sp. "Bicolored Jaws" is believed to be trophically specialized, feeding on the highly unusual diet of terrestrial amphipods (Binford 2001), while molecular gut content analysis (Kennedy *et al*. in prep(a)) shows that the diet of *T.* sp. "Emerald Ovoid" consists mainly of moths and flies. A similar example is found in East Maui, where a clade of *T. stelarobusta*, *T. trituberculata* and *T. eurychasma* occurs in sympatry. These three species are also quite morphologically distinct and inhabit highly complementary niches, i.e. with very little niche overlap (Blackledge *et al*. 2003, Kennedy *et al*. in prep(c)). In both cases – Oahu and Maui – the lineages could have diverged in allopatry due to the presence of geographic barriers caused by volcanic activity; but by the time these barriers receded, the species were different enough from one another to be reproductively isolated, and potential competition may have subsequently selected for further niche divergence. We see the same pattern in the Spiny Leg species of Kauai, which are monophyletic but which represent three of the four Spiny Leg “ecotypes” recognized by Gillespie (2004); and also in Oahu, where all Spiny Leg species except for *T. quasimodo* form a clade.

The other side of this phenomenon is the convergent evolution of morphological and ecological traits in multiple lineages within the radiation. Indeed, we recover repeated occurrences of every “ecotype” of Spiny Leg species – “large brown,” “green,” “small brown,” and “maroon” – in multiple different clades and across the archipelago. This is not a novel result, but matches earlier studies of the Spiny Leg clade (Gillespie *et al*. 1997; Gillespie 2004, 2005); the addition of >2000 bp of DNA data continues to support the finding that each of the four ecotypes evolved more than once. The main novelty of our study, then, is what we find in the web-building group, whose relationships have thus far been difficult to resolve. We find that, like the Spiny Leg clade, the web-builders appear to have evolved certain morphological phenotypes in multiple different lineages. The “Pointed Abdomen” and “Elongate Forest” phenotypes both have representatives on every island, with their closest relatives belonging to morphologically and ecologically distinct groups. The “Eurylike” phenotype occurs independently on Oahu, Maui Nui, and Kauai – and the Kauai lineage is so divergent from the rest of the web-building group that it may actually be closer to *T. hawaiensis*. Finally, the spiders described as *T. paludicola* appear to be polyphyletic, and the morphology of this “species” may have arisen as many as three separate times. While we do not yet know the order in which the different web-building morphologies evolved, and therefore cannot say which ones are plesiomorphic and which are apomorphic, this would be an intriguing avenue of study because it could reveal whether certain morphologies are adaptive for certain ecological niches. Identifying the ancestral morphological state of web-building Hawaiian *Tetragnatha* could thus contribute greatly to our understanding of evolution in this group.

Standing in contrast to these examples of apparent ecological diversification is the niche conservatism we see in some other lineages within the *Tetragnatha* radiation. The most striking example is the “Mountain Top clade,” which includes species from multiple islands – Kauai, Oahu, Maui and Hawaii Island – all of which are found only in dry forests. That these species are monophyletic, yet inhabit so many of the islands in the archipelago, indicates the importance of ecological (e.g. climatic) constraints in this group. While other lineages diversified into species that filled a wide range of new ecological niches, the “Mountain Top clade” appears to be adapted specifically to dry forest and consequently has been unable to establish itself in any other type of habitat. Hence, it appears that habitat filtering has played an integral role in the evolution of this clade.

Conclusions

The adaptive radiation of Hawaiian *Tetragnatha* has much to offer as a system for identifying the ecological and evolutionary factors at play in rapidly evolving lineages. We find evidence for contrasting evolutionary patterns: on one hand, clades can diverge into groups of ecologically and morphologically different species, leading to the coexistence of very closely related species in a habitat; on the other hand, a lineage may experience phylogenetic niche conservatism such that all of its species are confined to ecologically similar habitats, as appears to be the case with the dry forest specialists within the "Mountain Top" clade. While we cannot definitively say under which conditions these different lineages have evolved, we can speculate that such contrasting patterns of apparent niche divergence and niche conservatism, as well as convergent evolution of different ecotypes, likely reflect a mix of various evolutionary processes that have occurred over the history of the radiation.

Hawaii represents a range of stages in community assembly, from its youngest island (Hawaii Island at <1 my) to its oldest (Kauai at 5.1 my). This age-structured arrangement of islands – referred to as a "chronosequence" (Walker *et al*. 2010) – allows the examination of ecological and evolutionary dynamics at multiple different points in time, and the evolutionary patterns we see in *Tetragnatha* are especially interesting in the context of this age structure. In particular, the pattern of monophyly within islands – e.g. in the Kauai and Oahu Spiny Leg species, the clade of *T.* sp. "Bicolored Jaws" and *T.* sp. "Emerald Ovoid," and the three Maui Nui endemics within the "Eastern Forest" clade – does not occur on the youngest island. Given the ages of the Hawaiian islands, the relative lack of monophyly within the Big Island fits expectations: the island simply has not existed long enough for cladogenesis to have occurred in its endemic lineages. Maui, which is of intermediate age, contains one monophyletic group but also hosts a mix of species whose closest relatives are on different islands. Oahu, older than Maui, contains mostly Oahu endemics – the "Oahu Spiny Leg" clade, the "Emerald Ovoid"/"Bicolored Jaws" clade, and the clade of "Elongate Forest" species. Kauai, older still, possesses a clade of ecologically divergent Spiny Leg species. While the patterns in Kauai web-builders are less clear – the three known species (*T.* sp. "Waves," *T.* sp. "Elongate Forest" and *T. maka*) appear in very different parts of the phylogeny – this may also be due to Kauai's older age and, perhaps, more complex history.

Indeed, the potential complexity of these spiders' evolutionary history, and the number of past events that might never be "knowable," will likely always pose a challenge. However, our knowledge of this remarkable system is only increasing. The phylogenetic analysis presented here is a critical step forward in our growing understanding of how and why this extraordinarily diverse group of spiders exists on Hawaii, and what the *Tetragnatha* adaptive radiation can tell us about evolution in other systems.

Locus	F Primer	Sequence 5’ to 3’	R primer	Sequence 5’ to 3’	Amplicon length (bp)	Compartment	Protein-coding?
COI	ArF1[1]	GCNCCWGAYATRGCNTTYCCNCG	Fol-degen-rev[2]	TANACYTCNGGRTGNCCRAARAAYCA	467	mitochondrial	yes
16SrDNA (16S)	16SF3[3]	CGRTYTRAACTCAGATCATGTA	16SR1[3]	TRACYGTRCWAAGGTAGCATAA	401	mitochondrial	no
12SrDNA (12S)	12SF1[3]	NCHACTWTGTTACGACTT	12SR1[3]	AMTAGGATTAGATACCCT	424	mitochondrial	no
Cytochrome B (CytB)	CB3degB[3,7]	GAGGDGCHACHGTWATYACHAA	CB4deg[3,7]	RAARTATCATTCDGGTTGRATRTG	406	mitochondrial	yes
18SrDNA (SSU)	SSU_FO4[4]	GCTTGTCTCAAAGATTAAGCC	SSU_R22[4]	GCCTGCTGCCTTCCTTGGA	421	nuclear	no
18SrDNA (18SM)	18s_2F[5]	AACTTAAAGRAATTGACGGA	18s_4R[5]	CKRAGGGCATYACWGACCTGTTAT	351	nuclear	no
28SrDNA (28SM)	28s_3F[5]	TTTTGGTAAGCAGAACTGGYG	28s_4R[5]	ABTYGCTACTRCCACYRAGATC	363	nuclear	no
Histone H3 (H3)	H3aF[6]	ATGGCTCGTACCAAGCAGACVGC	H3aR[6]	ATATCCTTRGGCATRATRGTGAC	374	nuclear	yes
ITS2	5.8S3F[3]	ATCACTHGGCTCRYGGRTCGATG	28S2R[3]	TTCTTTTCCTCCSCTHANTDATATGC	436	nuclear	no
Actin	Actin_F2[8]	GAYTTYGARCARGARATGGCNAC	Actin_R1[8]	GRTCDGCAATNCCWGGRTACAT	271	nuclear	yes

Table 1: Markers used for phylogenetic analysis. Amplicon lengths include primer binding sites.

[1]Gibson *et al*. 2014; [2]Yu *et al*. 2012; [3]Krehenwinkel *et al*. 2018a; [4]Fonseca *et al*. 2010; [5]Machida and Knowlton 2012; [6]Colgan *et al*. 1998; [7]Barraclough *et al*. 1999; [8]This study

Subset	Marker(s)	Location in alignment	nst	Rates
1	Actin codon pos. 2, 18SM, SSU	271-495\3, 1-269, 796-1165	1	invgamma; statefreqpr=fixed(equal)
2	Actin codon pos. 1	270-495\3	6	gamma
3	H3 codon po. 3, Actin codon pos. 3	1529-1854\3, 272-495\3	1	propinv; statefreqpr=fixed(equal)
4	28SM	496-795	6	invgamma
5	12S	1166-1526	6	invgamma
6	H3 codon pos. 1	1527-1854\3	6	gamma
7	H3 codon pos. 2	1528-1854\3	6	propinv
8	ITS2	1855-2185	6	invgamma; statefreqpr=fixed(equal)
9	CytB codon pos. 1, COI codon pos. 1	2186-2541\3, 2909-3326\3	6	gamma
10	CytB codon pos. 2, COI codon pos. 2	2187-2541\3, 2910-3326\3	6	invgamma
11	CytB codon pos. 3	2188-2541\3	6	invgamma
12	16S	2542-2908	6	invgamma
13	COI codon pos. 3	2911-3326\3	6	propinv

Table 2: Best partitioning scheme and substitution models for concatenated dataset as determined by PartitionFinder.

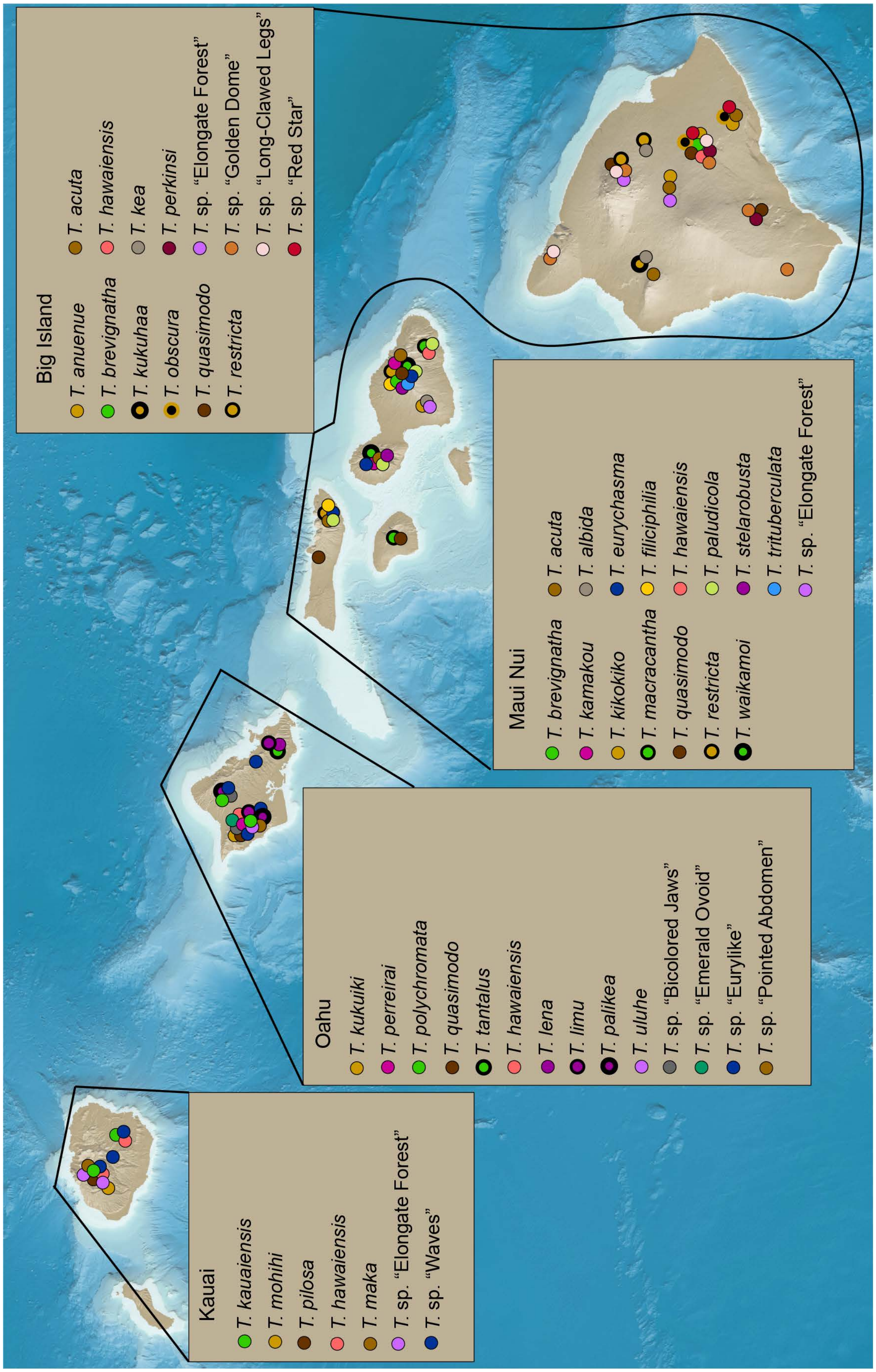

Figure 1: Map of the Hawaiian Islands, depicting localities of all specimens used in this study. Image adapted from Patterson (2014).

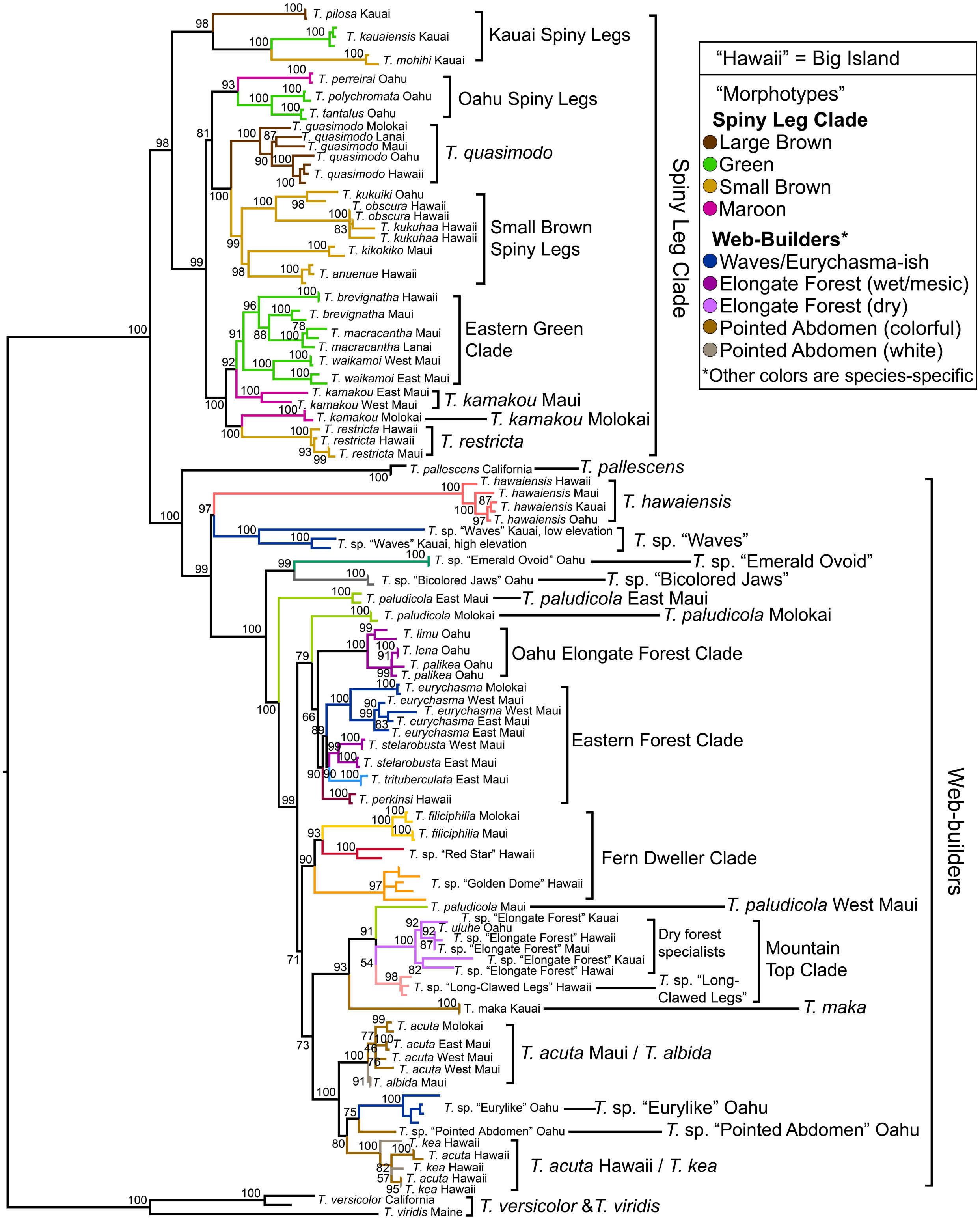

Figure 2: Maximum Likelihood (ML) phylogeny of Hawaiian *Tetragnatha* spp., generated using IQTree. Bootstrap support value for each node is depicted on the left side of the node. Branch colors represent "morphotype" or species.

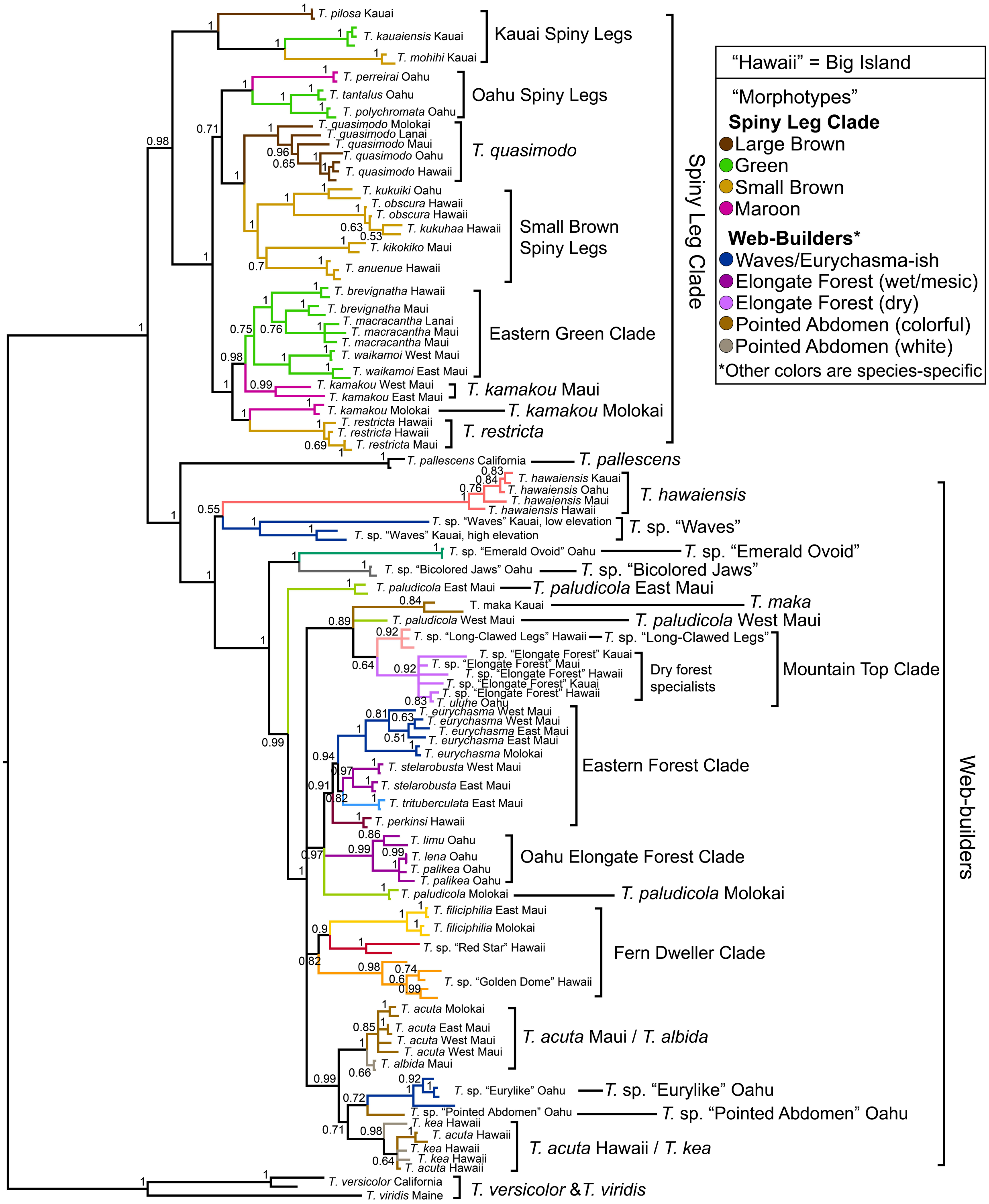

Figure 3: Bayesian phylogeny of Hawaiian *Tetragnatha* spp., generated using MrBayes. Posterior probability for each node is depicted on the left side of the node. Branch colors represent "morphotype" or species.

Chapter 4

Niches in time: molecular gut content analysis reveals changing ecological relationships among Hawaiian *Tetragnatha* spiders along a chronosequence

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Abstract

Understanding the ecological dynamics of adaptive radiation is a key step toward identifying the nature and timing of the evolutionary processes leading to such strikingly diverse systems. The adaptive radiation of Hawaiian *Tetragnatha* spiders presents an ideal study system for characterizing these ecological dynamics and their putative role in the evolutionary history of the lineage. Using Hawaii's geologic age structure as a chronosequence, it is possible to infer past ecological and evolutionary processes based on present-day patterns in younger habitats. We explore ecological niche dynamics across a temporal gradient in the Hawaiian *Tetragnatha*. We use a recently developed method for molecular gut content analysis to characterize the diets of 23 different *Tetragnatha* species belonging to two different clades, which correspond to fundamentally different hunting strategies, from three habitats of differing substrate age. We also test for effects of phylogenetic relatedness on trophic niche overlap between species. Based on taxonomic classifications of prey to both order and "operational taxonomic unit" (roughly comparable to species) levels, we uncover a complex and varied pattern of dietary niche relationships among *Tetragnatha* species. The two hunting strategies are associated with significantly different types of prey. In some lineages, we find apparent dietary difference between closely related species, whereas in others, diet appears to be phylogenetically conserved. We also find an apparent decrease in dietary overlap as island age increases, suggesting that species in older communities have evolved under selection for niche partitioning. Our results largely match expectations based on theory, which predicts that species in younger communities should exhibit high niche overlap while those in older communities should show pronounced niche differences.

Introduction

Adaptive radiation provides an ideal intersection for studies of evolution and ecology. The evolutionary processes that drive adaptive radiation result in complex and varied patterns of niche ecology within clades, generating systems in which evolutionary processes can be examined using replicate sets of species. The chief challenge to understanding evolutionary processes is the inability to look back in time to determine how diversification and adaptation occurred; instead, we must work with what we see now. However, one powerful tool that can illuminate the dynamics of these evolutionary and ecological processes is the "chronosequence," a temporally structured landscape in which present-day ecological and evolutionary patterns on younger sites can be used to infer historical processes on older sites (Walker *et al*. 2010).

The Hawaiian Archipelago is a classic example of a chronosequence, with its islands arranged sequentially from oldest in the northwest (Kauai, 5.1 my) to youngest in the southeast (Hawaii, < 0.7 my). The Hawaiian archipelago has formed – and continues to form – on a volcanic hot spot, and the shift of the underlying tectonic plate toward the northwest has resulted in this linear arrangement of islands. This geologic age structure, coupled with Hawaii's geographic isolation from other landmasses, make the archipelago a "natural laboratory" that is ripe with opportunity to test the timing, drivers, and results of evolutionary processes at different stages in time. Hawaii hosts a great number of adaptive radiations, some very well known (e.g., silverswords (Robichaux *et al*. 1990), honeycreepers (Lovette *et al*. 2002), and picture wing *Drosophila* (Magnacca and Price 2015)) and others only beginning to be understood (e.g. Xyleborine ambrosia beetles (Cognato *et al*. 2018), *Nesosydne* planthoppers (Goodman *et al.* 2012)).

It is generally agreed that adaptive radiation can occur when there is "ecological opportunity" – i.e., there are potential ecological niches available that are not yet occupied by likely ecological rivals (Stroud and Losos 2016). Theory predicts that as species arrive in habitats with ample ecological opportunity, they may expand their ecological niche such that it is broader than the niche in the ancestral range (Fukami 2015). As more and more species accumulate, either by subsequent colonization events or by cladogenesis, the available niche space is filled (Ricklefs 2010). This can lead to competition pressure, which in turn can lead to niche partitioning, the specialization of co-occurring species on different subsets of the available resources such that interspecific overlap in resource utilization is decreased or minimized (Schoener 1974). At relatively advanced stages of adaptive radiation, communities are therefore expected to exhibit a greater degree of niche partitioning – a lower degree of niche overlap – than evolutionarily younger communities. While the phenomenon of decreasing niche breadth over evolutionary time is often discussed in the literature (McPeek 2008, Gavrilets and Losos 2009, Gillespie and Baldwin 2010), it remains challenging to obtain direct evidence of this process having occurred.

Ecological niche consists of several potential axes (Leibold 1995, Kearney *et al*. 2010), including climate, elevation, microhabitat/substrate preference, and, importantly, diet. Given that food resources within a habitat are rarely unlimited, trophic ecology in particular is a promising avenue for exploring niche dynamics in animal communities. Following in the theoretical framework of ecological dynamics of adaptive radiation, communities are expected to show high levels of dietary overlap in the early stages of adaptive radiation, whereas later stages should show increasingly specialized diets with less overlap in food resource exploitation – in other words, increased trophic niche partitioning – among species. Hawaii offers numerous opportunities, in the form of its many adaptive radiations, to test this prediction; however, Hawaiian spiders in particular present a rich and tractable system for study. The Hawaiian spider fauna is relatively depauperate in regard to deeper taxonomy (i.e. genus level and above), yet of the genera that have become established on Hawaii, most have diversified into a multitude of different species – notably, *Mecaphesa* crab spiders (Garb and Gillespie 2009), *Ariamnes* stick spiders (Gillespie *et al*. 2018), *Orsonwelles* sheet web weavers (Hormiga *et al*. 2003), Theridiine cobweb weavers (Arnedo *et al*. 2007), and *Cyclosa* orb weavers (unpubl. data), among others. However, the best-studied spider lineage in Hawaii is the remarkable *Tetragnatha* long-jawed orbweavers, which constitute an adaptive radiation (Gillespie 2015).

Hawaii's >50 *Tetragnatha* species are markedly different from mainland *Tetragnatha* spiders in their morphology, ecology and behavior, and are divided into two major clades: one that retains the plesiomorphic hunting strategy of capturing prey in orb-shaped webs (Gillespie 1992, 1994, 2003), and another, known as the Spiny Leg clade, which has abandoned web-building and instead hunts actively (Gillespie 1991, 2002b). Within both clades, there is evidence for convergent evolution of ecological (Blackledge and Gillespie 2004) and morphological (Gillespie 2004) traits occurring multiple times across the islands. Among the 17 species in the Spiny Leg clade, four "ecomorphs" have been identified, each associated with a specific resting substrate type: "large brown" with bark, "green" with living leaves, "small brown" with twigs, and "maroon" with lichens and mosses. In the web-builders, less is known about whether particular morphologies are associated with microhabitats; however, there are certain morphological phenotypes that appear to occur in disparate parts of the phylogeny (Kennedy *et al*. in prep(b)), and convergence in the architecture of the web has been documented (Blackledge and Gillespie 2004).

The striking ecological and morphological diversity of Hawaiian *Tetragnatha* raises the intriguing question of how diet may have evolved alongside these ecological and morphological phenotypes. Although the diets of these spiders have been explored to a preliminary extent using field observation (Binford 2001, Blackledge *et al*. 2003), much remains to be discovered about the exact dietary compositions of a broader representation of *Tetragnatha* species across the archipelago. Using a recently developed method for molecular identification of gut contents (Krehenwinkel *et al*. 2017), we test how diet and trophic niche overlap change over evolutionary time within the Hawaiian *Tetragnatha*. We characterize the diets of 23 *Tetragnatha* species from islands of three different geologic ages: Hawaii, at < 0.7 my; Maui, at 1.3 my; and Oahu, at 3.4 my (Carson and Clague 1995). In our analysis we include species from both major clades of Hawaiian *Tetragnatha*, each of which corresponds to a different hunting strategy: the web-builders, and the actively hunting Spiny Leg clade (Gillespie 1991, 2002b).

We test the following hypotheses: 1) Spiders of different hunting types (web-building versus actively hunting) consume different taxonomic assemblages of prey due to the differences in their prey capture behavior – i.e., web-builders catch more flying prey whereas cursorial hunters catch more walking or crawling prey; 2) Diet is an important axis of ecological divergence in recently differentiated species, such that spiders will consume significantly different diets from their sister species and other very close relatives. At the same time, more distantly related species that exhibit morphological and/or ecological convergence are expected to consume similar diets to members of their own "ecotype" (Gillespie 2004); and 3) Community dietary ecology changes over time, such that spiders in younger communities will have broader niches and higher levels of dietary overlap while those in older communities will have narrower niches and higher complementarity, because sympatric species in older communities may have evolved under selection for niche partitioning due to competition pressure. Building on a substantial body of work on the ecological affinities, substrate preferences, and hunting behaviors of Hawaiian *Tetragnatha*, this study adds a critical component to our understanding of these spiders' natural history by describing the taxonomic composition of prey identified directly from the spiders' digestive tracts. Furthermore, by testing these ecological, evolutionary and temporal hypotheses in an exemplary group of spiders, we illuminate dynamics at play in different stages of adaptive radiation, building on our knowledge of how such extraordinarily diverse systems arise.

Methods

Specimen collection

Spiders were collected on four separate field trips in February, April and November of 2016, and June of 2017, from the following sites:

Hawaii / Big Island:[*]
Mauna Loa: along Stainback Highway (19.562°N, 155.272°W, ± 200 m), 1365-1380 m
Mauna Kea: Laupāhoehoe Natural Area Reserve (19.922°N, 155.301°W, ± 100 m), 1340-1370 m

Maui:
Upper Waikamoi Preserve: Boardwalk (20.780°N, 156.228°W, ± 50 m), 1860-1920 m
Lower Waikamoi Preserve: Maile Trail (20.801°N, 156.255°, ± 50 m), 1400-1470 m

Oahu:
Waianae Mountains: Mt. Ka'ala (21.505°N, 158.142°W, ± 200 m), 1180-1220 m

Specimens were collected from the vegetation by visually searching and by "beating," holding a stiff square of canvas underneath the vegetation while shaking or striking the foliage so that spiders fall off onto the sheet (Coddington *et al*. 1991). Each spider was captured directly into an individual 7-dram plastic snap cap vial (Thornton Plastics), then preserved in 99% ethanol and stored at -20° C to prevent DNA degradation. Spiders were visually identified to species under a microscope. Twenty-three *Tetragnatha* species, of which 13 are web-builders and 10 are members of the Spiny Leg clade (i.e. active hunters), were included in the study (Table 1).

Laboratory work

DNA was extracted from the opisthosoma (abdomen) of each spider using a PureGene Cell and Tissue Kit (Qiagen) according to the manufacturer's protocol, with a 6-hour cell lysis step at 55° C. DNA pellets were hydrated with 30-100 µL of DNA Hydration Solution (Qiagen), depending on the size of the pellet. DNA extracts were visualized on a 1.5% agarose gel to verify their quality, then were stored at -4° C. Although the spider opisthosoma contains more spider DNA than prey DNA (Piñol *et al*. 2014, Krehenwinkel *et al*. 2017), we avoided the problem of overabundant predator DNA by using a PCR marker that amplifies poorly in *Tetragnatha* spp. This resulted in an exceptionally high yield of prey DNA in the final PCR product, approximately 87% (all sequence reads excluding those that matched *Tetragnatha* spp. when searched on the NCBI database; more detail is provided below in "Sequence processing"). The marker was a *ca.* 211-base pair fragment of mitochondrial Cytochrome Oxidase I (COI), amplified using the primer pair ZBJ-ArtF1c/ZBJ-ArtR2c (Zeale *et al*. 2011) and the Qiagen Multiplex PCR Kit, with the following cycling

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[*] Please note that we use the names "Hawaii" and "Big Island" interchangeably to refer to this island.

protocol: denaturation for 15 minutes at 95°; 31 cycles of 94° for 30 seconds, 46° for 90 seconds and 72° for 90 seconds; and final extension for 10 minutes at 72°. Primers contained added Illumina "tail" sequences to allow for dual indexing of the PCR products, followed by Illumina sequencing, as described in Lange *et al*. (2014). After the initial PCR, the products were indexed in a second round of PCR using TruSeq indexing primers to bind barcode sequences to each sample. TruSeq forward and reverse index primers were selected such that each sample had a unique combination of barcodes. The thermocycler protocol for indexing was the same as the one used in the initial round of PCR, except that the annealing temperature was 56° C and only 6 cycles were run. Indexed PCR products were then pooled for approximately equal coverage according to concentration, which was determined by band strength after electrophoresis on a 1.5% agarose gel. Pools were made with a target coverage of 50,000 sequence reads per specimen in order to ensure sufficient representation of prey DNA. The pools were cleaned of residual primers with 1X Ampure Beads XP (Beckman Coulter), diluted to 4 nM concentration, and run on an Illumina MiSeq with V3 chemistry (600 cycles) and 2 x 300-bp reads, at the California Academy of Sciences' Center for Comparative Genomics (San Francisco, CA) and at QB3 (University of California, Berkeley).

Sequence processing

MiSeq runs were demultiplexed based on TruSeq index barcode combination, with each unique combination of barcodes corresponding to a single sample (i.e. spider specimen). Forward and reverse sequence reads were merged in PEAR (Zhang *et al*. 2014) and quality filtered such that ≥ 90% of the reads were required to have a minimum quality of Q30 (which denotes a maximum of one base pair error per thousand base pairs) using the FastQC function in FastX-Toolkit (Gordon and Hannon 2010). The merged, filtered reads were then converted to FASTA format with FastX-Toolkit. FASTA files were dereplicated, i.e. duplicate sequences were removed so that a single copy of each unique sequence was left, using USEARCH (Edgar 2010). The output files were then clustered into OTUs (operational taxonomic units) at 97% similarity using USEARCH, with a *de novo* chimera removal step included in the OTU clustering process. OTUs for all specimens were then searched against the NCBI database using Basic Local Alignment Search Tool (BLAST; Altschul *et al*. 1990) to identify their taxonomy, then organized into a table using the awk and sed programming languages in Unix. The resultant OTU table showed individual specimens as columns and OTUs as rows, with each cell containing the number of sequence reads belonging to a given OTU identified from the gut content of a given specimen. OTU rows also contained taxonomic information taken from the BLAST search. Based on this taxonomic information, all OTUs identified as *Tetragnatha* sp. were removed as predator DNA. Because many Hawaiian arthropods are not yet represented in the NCBI database, we were unable to reliably assign OTUs to finer-level taxonomy; instead, we chose to work at the taxonomic level of Order, which is coarse enough to be reliable in its accuracy of assignment, yet fine enough to be informative in terms of the "functional group" or guild of prey. We also retained a dataset at the OTU level, which roughly corresponds to species (Hebert *et al*. 2003). We performed all subsequent analyses at both the OTU level and the ordinal level of prey.

Data analysis

All spider specimens with fewer than 1000 total reads were excluded from the analysis. Of the remaining specimens, most individuals had between 10,000 and 25,000 total reads. To generate the ordinal-level dataset, OTUs were summed by taxonomic order based on the BLAST results. The OTU and order tables were both rarefied (proportionally subsampled) to 1000 total reads per specimen using GUniFrac (Chen *et al*. 2012) in R 3.5.0 (R Core Team 2018).

For very rare spider species (2-9 individuals in the dataset; 9 out of 23 species), all specimens were included in the analysis. For more abundant species, a random number generator was used to select a subsample of 12 individuals, and all others were discarded. The sample size of 12 was chosen because it was the minimum sample size among the 14 species not considered "rare" in this dataset. The subsampling was done to minimize any potential effects of sampling unevenness, because larger numbers of individuals may contain larger numbers of prey taxa in their guts; conversely, a smaller sample size may result in artificially low estimates of prey diversity due to chance. The final dataset contained 222 specimens (see Table 1 for sample sizes by species).

PERMANOVA was used to analyze differences in diet at both the ordinal and the OTU levels with the adonis function of the vegan package (Oksanen *et al.* 2018) in R. First, overall effects of island (Hawaii/Maui/Oahu) and hunting type (web-building/active hunting) were tested with PERMANOVA allowing for interaction between these variables. Then, because well-established post-hoc tests for PERMANOVA are not readily available, analysis was done separately for each pair of species in order to identify pairwise differences. Pairs of species were tested if they met at least one of the following criteria: 1) sympatry, i.e. occurring on the same island; 2) "sisters" or very close relatives according to the phylogeny presented by Kennedy *et al*. in prep(b); or 3) members of the same "ecotype" (e.g. "maroon" or "small brown;" Gillespie 2004). The results of these pairwise tests were used to assess patterns of trophic niche overlap and complementarity within islands, across the phylogeny, and over evolutionary time (i.e. with Hawaii representing the youngest and Oahu the oldest community); two species were considered to overlap in their trophic niche if PERMANOVA found no significant difference between the species' diets.

In addition, the standardized Levins' index of niche breadth (Hurlbert 1978) was calculated in Excel Version 1808 (Microsoft Office 2016) for each species using the equation:

$$B_A = \frac{\left(\frac{1}{\sum_{i=1}^{n} p_j^{\,2}}\right) - 1}{n - 1}$$

where B_A is the niche breadth, n is the number of prey types, and p_j is the proportion of prey reads belonging to the jth prey found in a given spider species' gut. This index was calculated at both the OTU and the ordinal levels of prey for all spider species in order to test the hypothesis that niche breadth decreases as island age increases. A two-way ANOVA was run in R to test for effects of island (i.e. age), hunting strategy, and island x hunting strategy interaction on Levins' niche breadth.

To visualize niche relationships among the spiders, Principal Coordinates Analysis (PCoA) plots, which show the ways in which different species' diets cluster together or are distinct from one another, were generated based on Bray-Curtis distances between spiders' diets using the ecodist (Goslee and Urban 2007) and ape (Paradis *et al.* 2004) packages in R. For clarity of presentation, specimens were pooled together by species and then each species was rarefied to 1000 reads so that each species was represented by a single point on the plot.

Results

OTU-clustering of prey reads yielded 436 separate OTUs. From these, 10 arthropod orders were identified. The orders, and the proportion of the final dataset they occupied, were: Diptera (38%), Lepidoptera (23%), Hemiptera (21%), Araneae (5.9%), Hymenoptera (4.9%), Collembola (1.9%), Psocoptera (1.8%), Acari (1.3%), Coleoptera (1.2%), and Neuroptera (0.04%). Fig. 1 shows a bipartite network of associations between these prey orders and the *Tetragnatha* spiders, grouped by island and hunting type. Interaction strengths, represented by the width of links between predator and prey, show that Diptera is a substantial component of prey in web-building spiders, whereas Hemiptera is a large component of Spiny Leg spiders' prey. Lepidoptera is abundant in the diets of both web-building and Spiny Leg spiders. The remaining orders make up smaller proportions of the spiders' diets.

Relationships between dietary niches of species across the Hawaiian chronosequence

Tetragnatha diets, based on taxonomic composition of gut contents, showed patterns of differentiation by both island and hunting type (Fig. 2). "Hunting type" is either web-building or Spiny Leg (actively hunting), and is expected to show an effect on spiders' diets under Hypothesis 1. "Island" is used to represent the age of the community in which a given spider occurs (Hawaii being the youngest and Oahu the oldest); according to Hypothesis 3, species in older communities are expected to have narrower dietary niches. At the OTU level, a significant effect of island, hunting type, and island x hunting type interaction was found on diet (all $p = 0.001$), supporting Hypothesis 1 which predicts significant dietary differences between web-builders and Spiny Leg spiders. At the ordinal level, island did not have a significant effect on diet ($p = 0.092$), but hunting type and island x hunting type interaction remained significant predictors of diet ($p = 0.001$), also supporting Hypothesis 1. At the OTU level, spiders were generally clustered by island; in particular, the Big Island appeared quite separate from Maui and Oahu (Fig. 2). At the ordinal level, islands were overlapping, but spiders largely grouped by hunting type (web-building vs. active hunting). However, at both OTU and ordinal levels, *Tetragnatha* diets showed slightly different patterns on each island (Fig. 3). Dietary "overlap" between a pair of species is defined here as *not* having been found to differ significantly by PERMANOVA, and relationships found *not* to differ significantly are indicated by dotted lines (Fig. 3), while significant differences are shown simply by the absence of any line connecting a given pair of species. On the Big Island, diet was associated primarily with hunting style: web-building spiders and Spiny Leg spiders fell in separate places in the PCoA space, and most pairwise comparisons between members of different hunting types showed significant differences ($p \leq 0.05$; Fig. 3A). On Maui, there was less of a dietary distinction between hunting types, and increasing dietary overlap between web-builders and Spiny Leg spiders (Fig. 3B). On Oahu, although only a few species are represented in our analysis, all of these species appeared to consume different diets from one another ($p \leq 0.05$ for all comparisons except, at the OTU level, *T.* sp. “Emerald Ovoid” and *T.* sp. “Eurylike”; Fig. 3C)) – in other words, almost no trophic niche overlap was found in the oldest site.

Dietary divergence and convergence in a phylogenetic context

Pairs of sister species or very close relatives (nearly sisters) were compared to test whether dietary niche was significantly different between close relatives: in other words, whether diet was an axis of evolutionary divergence. In the “Eastern Forest” clade, we found significant dietary differences at both OTU and ordinal levels between the sisters *T. stelarobusta* and *T. trituberculata* ($p = 0.004$ at OTU level, $p = 0.05$ at ordinal level; Fig. 4). *T. eurychasma*, the nearest relative to this sister pair, had a similar diet to *T. stelarobusta* ($p = 0.335$ at OTU level, $p = 0.113$ at ordinal level) but a different diet from *T. trituberculata* at the ordinal level ($p = 0.041$). In the “Fern Dwellers,” the sister pair of *T. filiciphilia* and *T.* sp. “Red Star” had significantly different diets at the OTU level ($p = 0.003$) but not at the order level ($p = 0.096$), while *T.* sp. “Golden Dome” consumed a similar diet to *T.* sp. “Red Star,” with which it occurs sympatrically ($p = 0.342$ at OTU level, $p = 0.056$ at ordinal level), and a different diet from *T. filiciphilia* ($p = 0.027$ at OTU level, $p = 0.018$ at ordinal level). The next pair of close relatives, *T. acuta* and *T.* sp. “Eurylike,” were found to consume similar diets at the ordinal level ($p = 0.295$) but different diets at the OTU level ($p = 0.037$). In the “Eastern Green” clade of Spiny Leg spiders (Fig. 5), diet was generally conserved: we found no significant differences in prey order between Hawaii *T. brevignatha* / Maui *T. brevignatha* ($p = 0.122$), Hawaii *T. brevignatha* / *T. waikamoi* ($p = 0.121$), or Maui *T. brevignatha* / *T. waikamoi* ($p = 0.475$). However, *T. waikamoi* consumed a different assemblage of prey OTUs from either population of *T. brevignatha* ($p = 0.001$ for comparison with Hawaii population; $p = 0.007$ for Maui population). Similarly, the co-occurring and closely related Small Brown species *T. anuenue* and *T. obscura* had different diets at the OTU level ($p = 0.04$), but similar diets at the order level ($p = 0.455$). The two populations of *T. quasimodo* also showed differences in the OTU composition of their prey ($p = 0.001$), but at the order level, their diets were nearly identical ($p = 0.929$; Fig. 5; see also pie charts in Fig. 3A and 3B).

In addition to testing for dietary divergence among close relatives, we measured pairwise dietary differences in members of more distantly related “ecotypes” – “maroon,” “green,” “large brown” and “small brown” – which appear to have repeatedly evolved in the Hawaiian *Tetragnatha* radiation (Gillespie 2004). One of these pairs was *T. perreirai* / *T. kamakou*, representing the “maroon” ecotype of Spiny Leg spiders (Fig. 5). *T. perreirai* and *T. kamakou* had marginally different diets at the order level ($p = 0.052$), but significantly different diets at the OTU level (p =

0.001). We also compared *T. restricta*, a “small brown” species, with the relatively distantly related *T. anuenue* and *T. obscura* (both of which are also “small brown” species). Significantly different diets (p = 0.001) were found at OTU and ordinal levels for both of these comparisons. Finally, we compared two web-building species (Fig. 4) belonging to the putative ecotype “eurychasma-like”: *T. eurychasma* and *T.* sp. “Eurylike,” which is most closely related to *T. acuta* (Kennedy *et al.* in prep(b)). *T. eurychasma* and *T.* sp. “Eurylike,” which are characterized by similar morphologies (small, greenish, with a distinctive dark wave-shaped marking on the lateral abdomen), were found to have similar diets to one another at both the OTU (p = 0.202) and the order level (p = 0.742; Fig. 4). Taken together, these comparisons showed no consistent pattern of trophic niche overlap correlating with convergently-evolved “ecotype,” although this pattern was found in the *T. eurychasma* / *T.* sp. “Eurylike” pair.

Trophic relationships across evolutionary time: overlap and complementarity within islands

Pairwise PERMANOVA was used to identify dietary differences and similarities among pairs of co-occurring spider species on each island (Fig. 3), with “island” representing the age of each community (Big Island < 0.7 my, Maui = 1.3 my, Oahu = 3.4 my). This analysis was performed to test Hypothesis 3, which predicted that species in younger communities (i.e. the Big Island) would show higher levels of overlap in their trophic niche than species in older communities (Maui, followed by Oahu), which were expected to have narrower niches.

On the Big Island (Hawaii), most Spiny Leg species appeared to consume different prey from one another at the OTU level, but similar prey at the ordinal level (Fig. 3A). *T. restricta*, which fed primarily on Lepidoptera and Hemiptera, was unusual in that its diet differed significantly from all other sympatric Spiny Leg species except for *T. quasimodo.* In contrast, web-builder diets largely overlapped with each other; the only exceptions were that *T.* sp. “Long-Clawed Legs” consumed a significantly different OTU assemblage from the other Big Island web-builders, and *T.* sp. “Golden Dome” consumed a significantly different diet from *T. perkinsi* at the order level. The Big Island showed a particularly striking pattern of dietary differentiation by hunting type at the ordinal level of prey: In web-builders, Diptera made up a large proportion of the diet (72% on average for the 5 species), whereas Spiny Leg spiders had a substantial contribution of Hemiptera (41% of the diet) and only 8% of their diet came from Diptera.

On Maui, there was no longer a clear differentiation between web-builder and Spiny Leg diets (Fig. 3B). We found a high OTU complementarity in Spiny Leg spiders: OTU compositions of both *T. brevignatha* and *T. waikamoi* diets were unique (i.e. significantly different from all others) among Maui Spiny Leg species. At both the OTU and the ordinal levels, Spiny Leg species shared more common dietary components with web-building species than with each other. In web-builders, *T. eurychasma* shared OTUs with – i.e. did not differ significantly from – all other sympatric species; *T. stelarobusta* and *T. filiciphilia* were most similar to each other, while *T. trituberculata* shared OTUs with (did not differ significantly from) *T. kamakou* and *T. quasimodo*. At the ordinal level, all web-builders overlapped with *T. eurychasma* except for *T. trituberculata*, which was unusual: *T. trituberculata* shared a similar diet to all sympatric Spiny Leg species, but differed significantly from all fellow web-builders except for *T. acuta*. *T. trituberculata* also had a higher proportion of Hemiptera prey than the other web-builders (41% of total prey; Fig. 3B pie charts). Again, *T. filiciphilia* and *T. stelarobusta* shared similar diets, which were significantly different from the diets of *T. acuta* and *T. trituberculata*. The diets of *T. filiciphilia* and *T. stelarobusta* were composed of approximately 50% each Diptera and Lepidoptera. Thus, overall, patterns of dietary overlap and complementarity on Maui did not show the same clear separation between web-builders and Spiny Leg spiders as was found on the Big Island.

On Oahu (Fig. 3C), all pairwise species comparisons showed significant dietary differences, except for the OTU compositions of the web-builders *T.* sp "Emerald Ovoid" and *T.* sp. "Eurylike," which showed a marginal difference (p = 0.063). The web-builder *T.* sp. "Bicolored Jaws" had a high contribution of Collembola (90%), which was very unusual, but these data should be regarded with extreme caution due to the low sample size of *T.* sp. "Bicolored Jaws" (n=2). The diet of *T.* sp. "Emerald Ovoid" was mostly Lepidoptera (65%), whereas that of *T.* sp. "Eurylike" was mostly Diptera (77%). The Spiny Leg species *T. perreirai* had a relatively high richness and evenness of orders in its diet, with nearly equal contributions of Hemiptera, Diptera, Araneae and Lepidoptera. Hence, diets showed high complementarity both within and between hunting strategies on Oahu, although these results should be taken with the caveat that we only had data for four species on this island.

The standardized Levins' index of niche breadth (Hurlbert 1978) was calculated for each spider species (Fig. 6), and putative effects of island (i.e. community age) and hunting strategy were tested with a two-way ANOVA. No significant effect of island, hunting strategy, or island x hunting strategy interaction was found at the OTU level. At the ordinal level, a significant effect of hunting strategy was found (p = 0.0007), where web-builders had significantly smaller niche breadth than Spiny Leg species (Fig. 6B), but there was no significant effect of either island or island x hunting type interaction. Thus, we found no pattern of change in niche breadth with community age.

Discussion

Our results show a complex and intriguing interplay of island age, hunting strategy and community composition in the dietary ecology of the Hawaiian *Tetragnatha*.

Hunting strategy and trophic niche

We find a strong effect of hunting type – web-building versus active hunting (i.e. Spiny Leg) – on diet (p = 0.001 at both OTU and ordinal levels), supporting Hypothesis 1. This effect is also apparent in the ways the web-builders versus the Spiny Leg species fall on the PCoA space (Fig. 2, Fig. 3). Our results suggest that hunting strategy strongly influences the "functional group" of prey that are caught and consumed, even if there are also some finer-scale differences at the species (OTU) level between spiders of the same hunting type (e.g. *T. anuenue* and *T. restricta* versus other Big Island Spiny Leg species; Fig. 3). It is reasonable to expect an effect of hunting type on prey capture. Aerial webs are very effective tools for intercepting aerial prey, such as flies and moths, whereas the active hunting strategy used by Spiny Leg species is probably better suited for capturing prey that walks or crawls on the substrate. This may explain the relatively high occurrence of Diptera (flies) in the guts of web-builders and of Hemiptera (true bugs) in the guts of Spiny Leg spiders. Interestingly, Lepidoptera make up an approximately equal proportion of the diets of web-builders and Spiny Leg spiders (Fig. 1). This may be explained by ontogenetic partitioning of the prey, whereby Spiny Leg spiders feed on the larvae (caterpillars) and web-builders feed on the adults (moths). Indeed, an observational study of prey collected directly from spiders' jaws found a high proportion of caterpillars (27% of total prey) and few adult moths (11%) being eaten by Spiny Leg spiders, versus a large proportion of moths (39% of prey) and no caterpillars being eaten by web-builders (Binford 2001). While our method does not allow for the identification of prey life stages, the fact that we still find significant taxonomic differences between the diets of web-builders and Spiny Leg spiders shows that hunting strategy plays an important role in determining a spider's trophic niche.

Diet and evolution: divergence versus niche conservatism

Our examination of diets in sister and near-sister pairs (Hypothesis 2) shows a complicated story. Some sister pairs support our hypothesis that closer relatives will show stronger ecological divergence from one another; others, however, show no significant difference between sisters. One group that does appear to show niche divergence in close relatives is the "Eastern Forest" clade. We find that *T. stelarobusta* has a significantly different diet from its sister, *T. trituberculata*. Interestingly, we find no difference between the diet of *T. stelarobusta* and that of another near relative, *T. eurychasma*; however, *T. eurychasma*'s diet is different from that of *T. trituberculata.* This suggests that perhaps *T. trituberculata* is the one whose diet has changed the most from a relatively plesiomorphic diet shared by *T. eurychasma* and *T. stelarobusta*. Indeed, the diet of *T. trituberculata* is more similar to that of the four co-occurring Spiny Leg species than to other web-builders on Maui (Fig. 3). *T. eurychasma*, *T. stelarobusta* and *T. trituberculata* occur sympatrically, so they could potentially compete for resources – and in fact, there is evidence for potential niche divergence in terms of web structure, microhabitat and stable isotope signatures in these species (Kennedy *et al*. in prep (c)). As a side note, *T. stelarobusta* and *T. filiciphilia*, which share overlapping diets at both OTU and order level, were also found to have the highest δ^{15}N ratios and web silk density among East Maui *Tetragnatha* species (Kennedy *et al*. in prep(c)), suggesting that there is a connection between web silk density and the taxonomic composition of prey that are captured in the web.

While the "Eastern Forest" clade supports our niche divergence hypothesis, other sister pairs do not. In the "Fern Dweller" clade, *T. filiciphilia* consumes a similar diet to its sister, *T.* sp. "Red Star," at the ordinal level (Fig. 4). By contrast, *T.* sp. "Golden Dome," the sister to the clade of *T. filiciphilia* / *T.* sp. "Red Star," has a significantly different diet from *T. filiciphilia*. However, *T.* sp. "Golden Dome" shares a similar diet to *T.* sp. "Red Star," even though the two species are sympatric. This may be an indication that these two species, which are endemic to the youngest island (Hawaii), have not coexisted for long enough to have undergone niche partitioning. If this were the case, it would fit with current theory on the ecological dynamics of adaptive radiation (Gavrilets and Losos 2009, Weber *et al*. 2017). Two other close relatives that show no significant dietary divergence are *T. acuta* and *T.* sp. "Eurylike," which have similar diets at the ordinal level. This may indicate that trophic niche is conserved within this lineage, though analysis of additional species within the immediate clade would be necessary in order to test this. Additionally, because *T. acuta* and *T.* sp. "Eurylike" occur on separate islands, they may simply not be under selection to diverge ecologically from one another. Spiny Leg species within the "Eastern Green" clade (represented by *T. waikamoi* and the Maui and Big Island populations of *T. brevignatha*) also show little to no evidence of trophic niche divergence: no significant differences at the ordinal level were found for any species pair within this clade (Fig. 5). Similarly, *T. obscura* and *T. anuenue* show no order-level differences in prey consumed, although their diets do appear different at the OTU level. However, *T. obscura* and *T. anuenue* are Big Island species, and as such belong to a young community in which niches may still be expected to overlap. Finally, the two analyzed populations of *T. quasimodo* show no dietary divergence at the ordinal level, and indeed, their prey compositions are visibly similar to one another (Fig. 3A and 3B, pie charts). This suggests that diet is relatively conserved within this species, even on different islands. These mixed results do not allow us to draw any conclusions regarding the effect of phylogeny on trophic niche: although we see strong dietary divergence in the "Eastern Forest" clade on Maui, other pairs of sisters or close relatives do not show significant differences at the ordinal level.

Just as our sister species comparisons show mixed results, so does our exploration of diet as a function of morphological ecotype. In the Spiny Leg clade, all of the species pairs we tested – *T. perreirai* / *T. kamakou*, *T. anuenue* / *T. restricta*, and *T. obscura* / *T. restricta* – showed significant differences in prey composition. In other words, belonging to the same ecotype does not necessarily mean sharing a similar diet, even at a relatively deep taxonomic level (order) of prey, and this means we found no evidence for diet evolving convergently along with morphology in these species. Perhaps some of these species pairs have finer-scale differences in their microhabitat than the known categories of "lichen/moss" (for *T. kamakou* and *T. perreirai*), "green leaves" (*T. brevignatha* and *T. waikamoi*), or "twigs" (*T. anuenue*, *T. obscura* and *T. restricta*), which could influence the types of prey that the spiders regularly encounter. Alternatively, perhaps the spiders are driven toward different dietary selective peaks by congeners in their communities, where there may be selective pressure to consume a different diet from co-occurring species (i.e. niche partitioning), but these selective peaks may vary depending on the specific composition of *Tetragnatha* within a given community.

In contrast to the Spiny Leg ecotypes, the putative ecotype "eurychasma-ish" (web-building) does appear to have a characteristic diet, at least in the two species we analyzed here. *T. eurychasma* and Oahu *T.* sp. "Eurylike" had similar diets at both the OTU and the order levels. This result is actually somewhat surprising, despite the morphological similarity of these two species, because the two species build different webs: Blackledge and Gillespie (2004) found that *T.* sp. "Eurylike" spins a more silk-dense web, similar to that of *T. filiciphilia*, than does *T. eurychasma*. Perhaps this silk density has only a negligible effect on the types of prey captured by *T.* sp. "Eurylike" and

T. eurychasma, or perhaps other, unknown, factors are swamping out any such effect. However, it is also possible that the small sample size of *T. eurychasma* (n = 4) skewed the PERMANOVA comparisons with other species, so any results relating to *T. eurychasma* should be regarded with some caution.

Trophic niche relationships across time

We find a clear separation of the diets of web-builders and active hunters on the youngest island (Hawaii, < 0.7 my; Fig. 3A). Things are not so separated on Maui, which is older than Hawaii (1.3 my). On Maui, some species overlap more with members of the other hunting type (active hunting versus web-building) than with their own hunting type (Fig. 3B). Overlap of web-builders and active hunters may reflect consumption of prey at different life stages: for example, Spiny Leg spiders may hunt caterpillars while web-builders intercept adult moths with their webs. Evidence of such prey partitioning by life stage has been documented on Maui (Binford 2001). This pattern on Maui is expected: over time, members of the same “functional group” (hunting type) may be under competitive pressure and therefore be selected to consume different prey from one another. Indeed, there is evidence for niche partitioning of microhabitat and via web structure in the Maui *Tetragnatha* (Blackledge *et al*. 2003, Kennedy *et al*. in prep (c)). On Hawaii, by contrast, perhaps there has not been sufficient time for such pressures to act on the evolution of the spiders there. We also find almost no overlap among spiders’ diets on Oahu, the oldest island we include in this analysis (Fig. 3C). This supports Hypothesis 3, which predicts higher levels of dietary overlap in the youngest sites, with decreasing overlap in older communities. Perhaps in Oahu, the apparently high levels of dietary complementarity among *Tetragnatha* have resulted from the relatively longer timespan over which these spiders have coevolved. However, it should be noted that our Oahu samples include only a few species, and hence we may be missing some of the complexity of ecological dynamics occurring on this island. Nevertheless, the lack of overlap on Oahu, coupled with the patterns on Hawaii and Maui (in particular, the apparent decrease in dietary overlap among species *within* each hunting type), appears to support our predictions under Hypothesis 3.

However, in terms of niche breadth *per se* (as opposed to dietary overlap/complementarity), we see no effect of island age: spiders’ niches are apparently no broader on Hawaii than they are on Maui or Oahu (Fig. 6). Although this result does not match our predictions, it also does not necessarily contradict the possibility that community (island) age has an effect on niche dynamics. While individual species’ niches might not become narrower over evolutionary time – or perhaps, at least not at the evolutionary timescale we examined – this does not mean that the *relationships among* different species’ niches do not change over time. Perhaps in the Hawaiian *Tetragnatha*, niches have not narrowed but have instead shifted such that each species feeds on a slightly different assemblage of prey, leading to the ecologically diverse communities of *Tetragnatha* we now see on Maui and Oahu.

Conclusions

We find intriguing and complex patterns of temporal ecological dynamics within the context of adaptive radiation. In our youngest site, differences in species' diets appear to correspond to broad categories – in this case, hunting strategy – while finer-scale interspecific differentiation is minimal. Even between very closely related species, such as *T.* sp. "Golden Dome" and *T.* sp. "Red Star," no significant dietary difference is found. However, this pattern changes with increasing island age. Hunting strategy becomes less important in determining diet, and there are more apparent dietary differences among closely related species that share a hunting strategy.

While our results for sister-species comparisons are not straightforward, they draw attention to the fact that dietary divergence can occur over very short evolutionary time: the differences between *T. stelarobusta* and *T. trituberculata* illustrate the apparent rapidity with which diet can change in a lineage. This lability reinforces the importance of diet as an axis of niche divergence and a worthy subject of study in other systems.

The increasing ease and efficiency with which molecular gut content analysis can be accomplished has opened up a world of possibility for the study of trophic interactions. While this approach has its limitations – we can identify the taxonomy of the prey, but not its life stage or other potentially relevant information – molecular gut content analysis still offers tremendous power for generating ecological data at both broad and fine scales. As the method continues to develop, and our genomic databases continue to improve with the addition of more and more taxa, it will eventually be feasible to characterize the diets of the entire Hawaiian *Tetragnatha* radiation – not just at the OTU and ordinal levels, but at family, genus and species levels – with high reliability, further elucidating the ecological and evolutionary processes that drive adaptive radiation. The work presented here is only a piece of the intricate puzzle of how the *Tetragnatha* radiation came to be; however, it represents a significant step forward in our understanding of the biological and ecological relationships within this amazingly diverse group of animals, and their implications for ecological and evolutionary processes in other systems.

Island	Hunting Type	Species	Sample Size
Hawaii (< 0.7 my)	active	*T. anuenue*	12
		T. brevignatha	12
		T. obscura	4
		T. quasimodo	12
		T. restricta	12
	web-building	*T.* sp. “Golden Dome”	12
		T. hawaiensis	12
		T. sp. “Long-Clawed Legs”	5
		T. perkinsi	12
		T. sp. “Red Star”	8
Maui (1.3 my)	active	*T. brevignatha*	12
		T. kamakou	12
		T. quasimodo	12
		T. waikamoi	12
	web-building	*T. acuta*	9
		T. eurychasma	4
		T. filiciphilia	12
		T. stelarobusta	12
		T. trituberculata	7
Oahu (3.4 my)	active	*T. perreirai*	12
	web-building	*T.* sp. “Bicolored Jaws”	2
		T. sp. “Emerald Ovoid”	8
		T. sp. “Eurylike”	7

Table 1: List of species included in the gut content analysis.

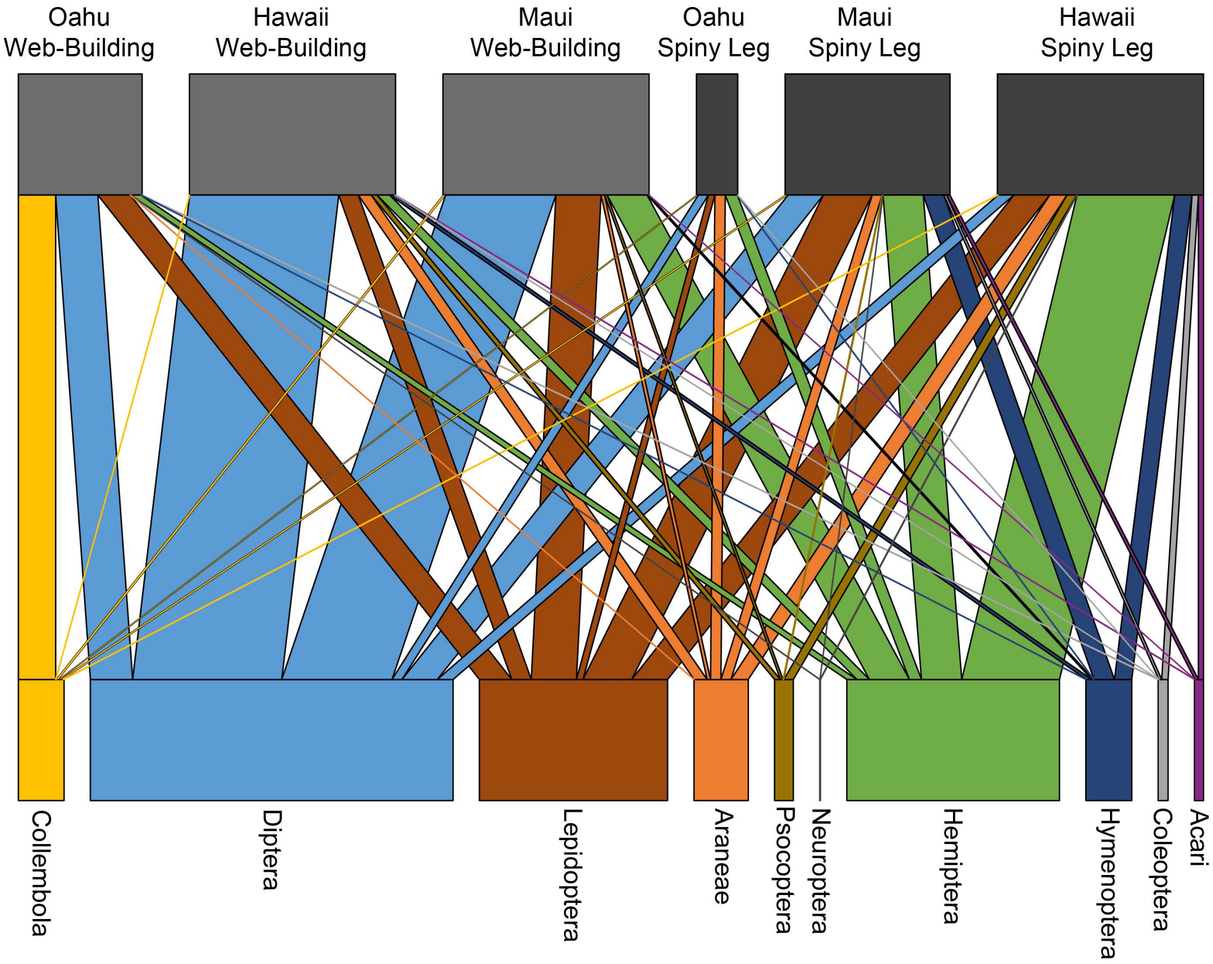

Figure 1: Bipartite network of predator-prey interactions. Prey are grouped by order, and spiders are grouped by island (Hawaii, Maui and Oahu) and hunting type (web-building and active hunting (Spiny Leg)). The width of bars connecting a predator to a prey type indicates interaction strength, based on number of sequence reads found in the predator's gut. Data were first rarefied to 1000 total reads per species of predator.

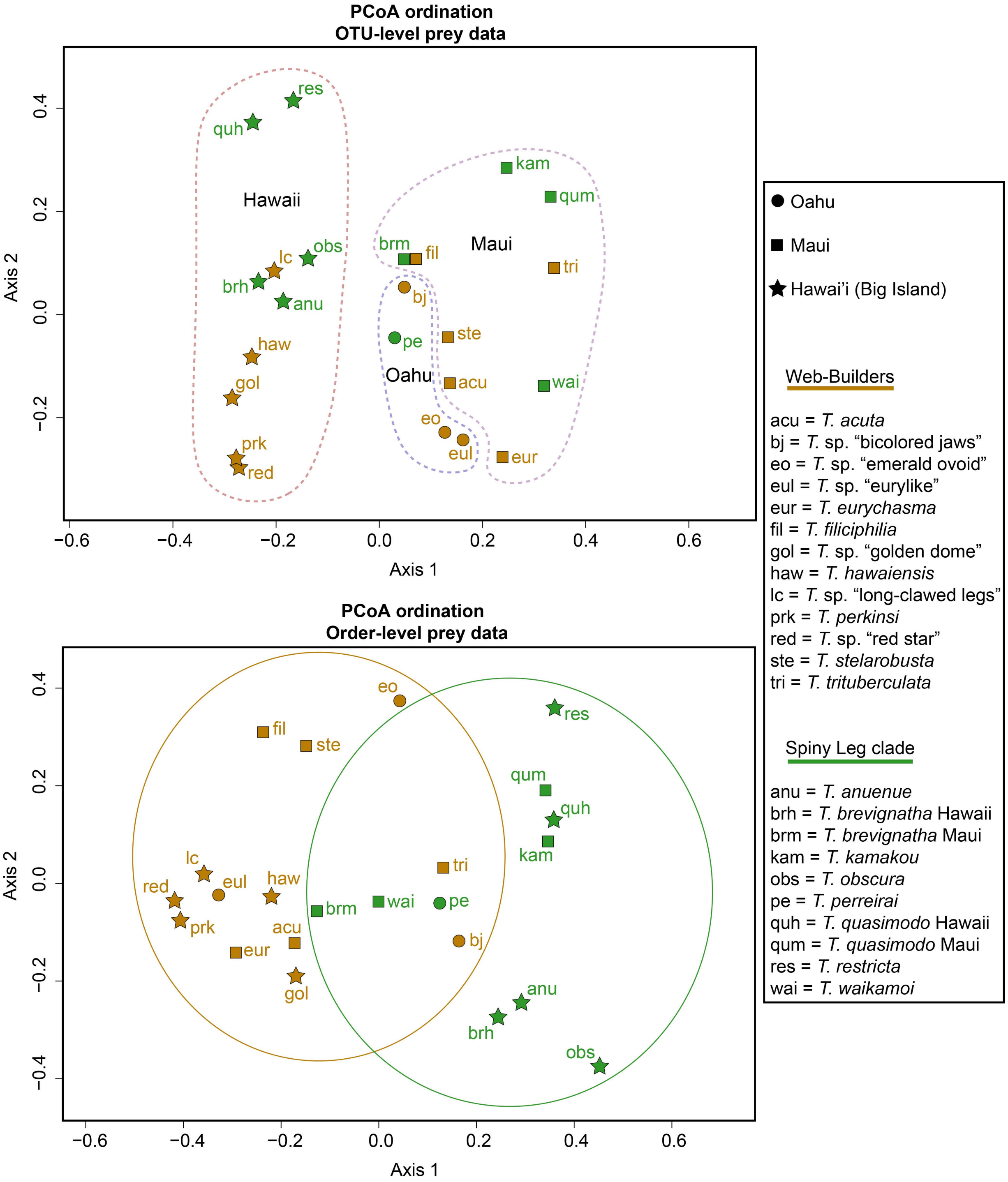

Figure 2: Principal Coordinates Analysis (PCoA) of spider diets for all three islands at both the OTU and ordinal levels. Data were first rarefied to 1000 total reads per spider species.

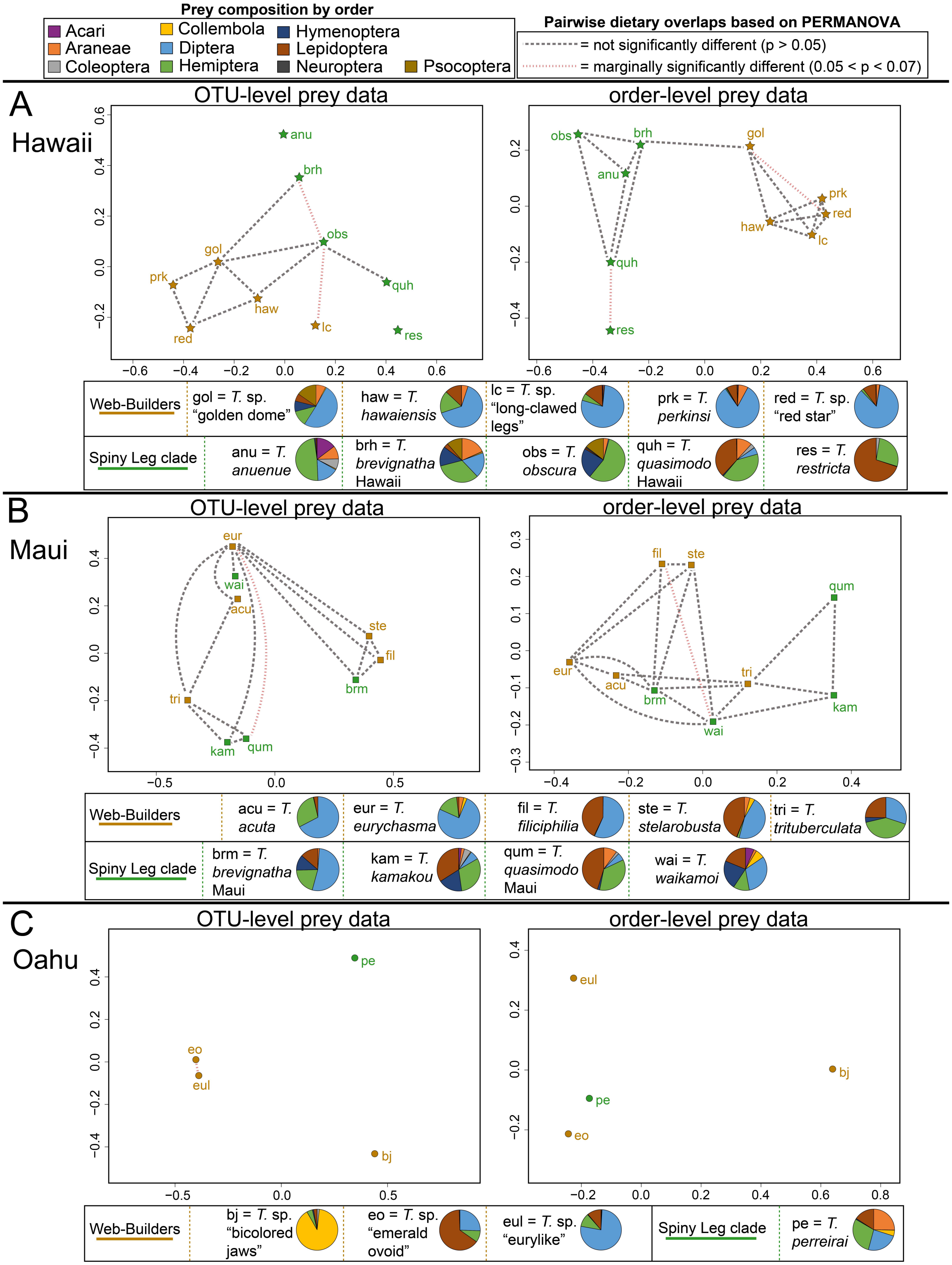

Figure 3: PCoA of spider diets, separated by island, at OTU and ordinal levels of prey. A gray dashed line indicates the absence of a significant dietary difference between two species, based on PERMANOVA. A pink dotted line indicates a marginally significant difference. Species that are not connected by a line have significantly different diets. Data were first rarefied to 1000 total reads per spider species.

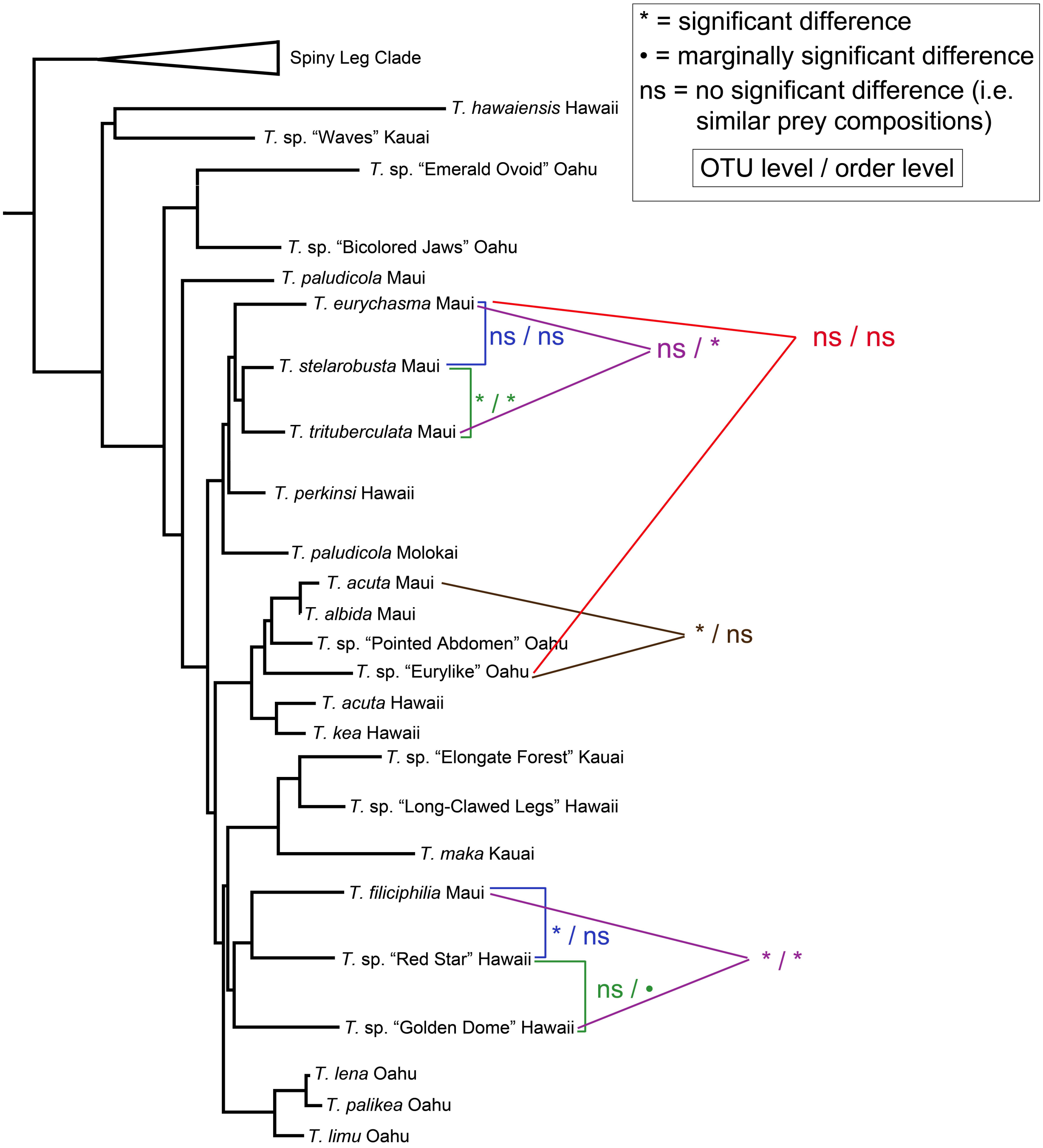

Figure 4: Phylogenetic relationships among web-builders, based on a recent phylogenetic analysis (Kennedy *et al*. in prep(b)). Colored lines connecting pairs of species indicate pairwise PERMANOVA analysis of dietary composition; results are indicated next to the connecting lines. ns = not significant, ● = marginally significant, * = significantly different. Significance is reported first at the OTU level / then at ordinal level.

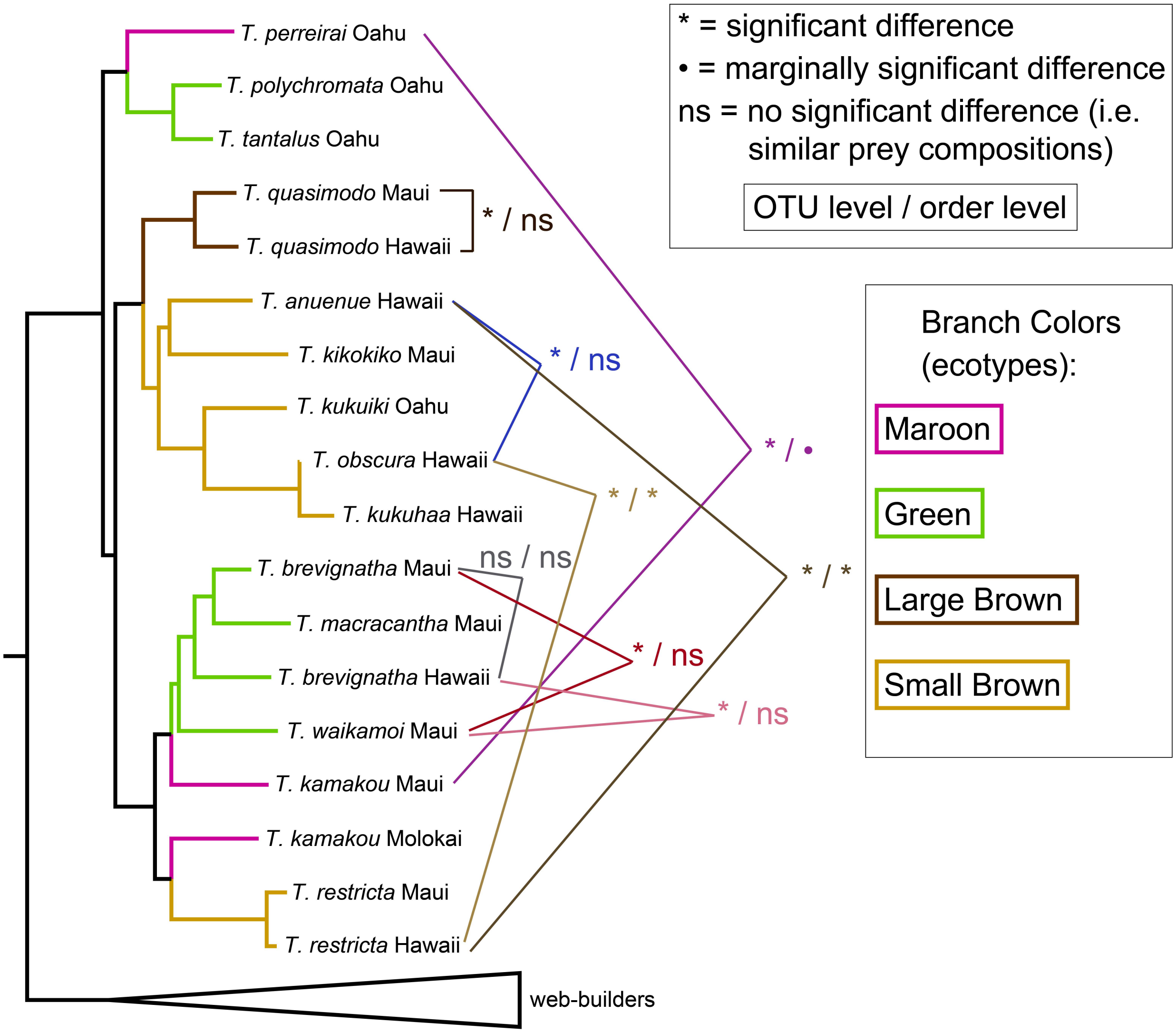

Figure 5: Phylogenetic relationships among Spiny Leg spiders, based on a recent phylogenetic analysis (Kennedy *et al.* in prep(b)). Colored lines connecting pairs of species indicate pairwise PERMANOVA analysis of dietary composition; results are indicated next to the connecting lines. ns = not significant, ● = marginally significant, * = significantly different. Significance is reported first at the OTU level / then at ordinal level. Branch colors indicate ecotype: fuchsia = "maroon," green = "green," dark brown = "large brown," goldenrod = "small brown."

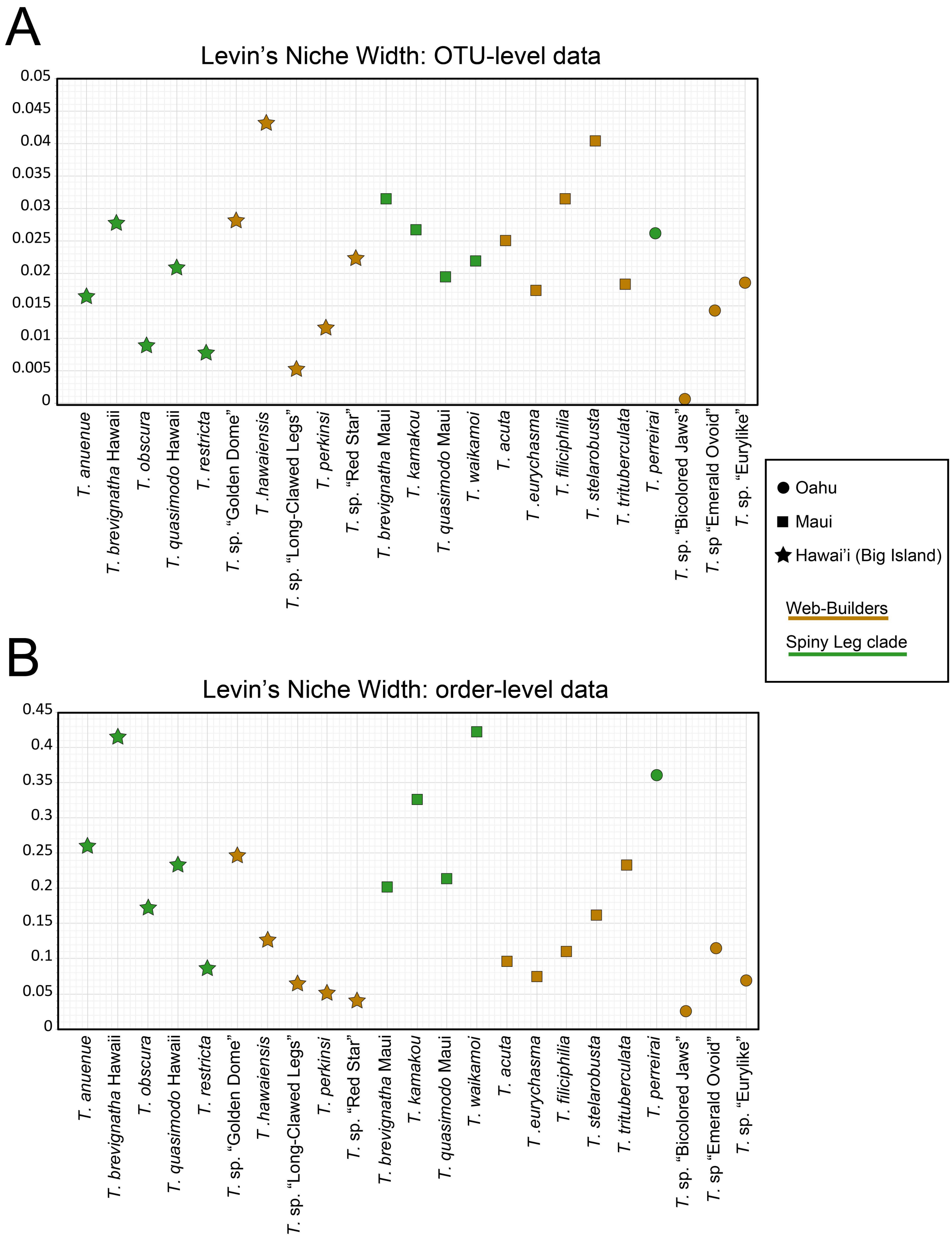

Figure 6: Standardized Levins’ index of niche width for each spider species, calculated at A) OTU and B) ordinal level.

Conclusion

The adaptive radiation of Hawaiian *Tetragnatha* has a tremendous amount to offer as a system for studying evolutionary ecology. In the course of this dissertation, I have found evidence for some of the phenomena that may have promoted evolutionary and ecological diversification in this lineage. In Chapter 1, I found that spiders closely reflect the chemical signatures of their substrates, and that such signatures are apparent even over distances as short as ~ 10 km. These results suggest severe dispersal limitation, which leads to reproductive isolation between populations and, ultimately, to evolutionary divergence. In Chapter 2, I examined ecological divergence within a community of *Tetragnatha* in East Maui. I discovered evidence for trophic niche partitioning at both the species level (among web-building species) and the broader level of hunting strategy (between web-builders and active hunters), suggesting that dietary differences may play a role in the coexistence of these species in their habitat. In Chapter 3 I offered an evolutionary framework in which to explore these ecological interactions by presenting a molecular phylogeny of every known species within the *Tetragnatha* adaptive radiation. Based on this phylogeny, I concluded that this adaptive radiation contains examples of divergent and convergent evolution, as well as, conversely, niche conservatism in one clade. Lastly, in Chapter 4, I explored trophic niche ecology on a habitat age gradient by characterizing the taxonomic compositions of spiders' gut contents on three islands. Based on these gut content data, I found complex and contrasting patterns of dietary divergence and conservatism in closely related species. In addition, I found changes in dietary niche over evolutionary time, which appeared to reflect niche shifts without any trend of increasing specialization.

Taken together, these results reveal a complex interaction of processes that potentially led to the present-day diversity of Hawaiian *Tetragnatha* spiders. No single phenomenon appears to apply to every group of species I examined: diet, for example, appears to be conserved in some sister pairs and divergent in others, and I find no convincing evidence that ecological morphotype determines a spider's diet. On the other hand, I do find a consistent pattern of higher δ^{15}N in web-building spiders than in the Spiny Leg clade, which demands an explanation. At present, the possibility that this isotopic difference is caused by a variable I did not measure – for example, silk production and/or recycling, or metabolic rate – cannot be discounted. And owing to the current limitations of DNA sequence databases, as well as character variation that molecular identification is unable to detect (e.g. life stage or specific ecological traits that are not shared with close relatives), I cannot confidently attribute this difference solely to prey composition, even though I found pronounced differences between web-builder and Spiny Leg diets. Needless to say, there is still a great deal of work left to do on this system.

As I imagine must be true for every biological system, the more we learn about the Hawaiian *Tetragnatha*, the more fascinating questions arise. At the same time, as we build on our knowledge of these spiders, and as technology and analytical methods continue to advance, an ever-increasing amount of data comes within our reach. The evolutionary trajectory of an animal lineage is shaped by a myriad of different ecological, morphological, physiological and behavioral traits. Colleagues are currently exploring the importance of mating behavior, chemical signaling, prey capture behavior, leg spine morphology, and other characters in generating the marvelous diversity of Hawaiian *Tetragnatha* that we see today, while others are working on characterizing entire genomes and building phylogenies for the whole *Tetragnatha* genus across its worldwide range. Dietary ecology is just one among several critical aspects of these animals' biology; and as we continue to build our knowledge, dietary ecology also promises to yield increasingly comprehensive and detailed information. One day I hope we will know what the webs of every *Tetragnatha* species look like, where they build them, what sorts of prey they capture and how this affects their isotopic signatures – and furthermore, *why* they behave in the ways that they do. Until then, every new discovery represents an important step forward in our understanding of how and why biodiversity is generated and maintained, not only in this system but in all life on Earth.

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Appendix A

Supplement to Chapter 3: List of specimens, and their localities, used in phylogenetic analyses of "A multilocus phylogeny of the adaptive radiation of Hawaiian long-jawed spiders (*Tetragnatha* spp)".

Specimen ID	**Species**	**Island**	**Locality**
RGG-C13_RndGldHaKip18GJ_13	anuenue	Hawaii	Kipukas
TPhy16-5F	anuenue	Hawaii	'Ola'a
GutPhy-5F	anuenue	Hawaii	Pu'u Maka'ala
RGG-240_SLHCbrevig10	brevignatha	Hawaii	Saddle Road
GutPhy-5G	brevignatha	Hawaii	Pu'u Maka'ala
TPhy16-11F	brevignatha	Maui	Lower Waikamoi Preserve, East Maui
TPhy16-1C	brevignatha	Maui	Lower Waikamoi Preserve, East Maui
TPhy16-11A	kamakou	Maui	East Maui
AshSus-4G	kamakou	Molokai	Kamakou Preserve
RGG-470_kamakou_Molokai_1	kamakou	Molokai	Kamakou Preserve
RGG-473_kamakou_W._Maui_2	kamakou	Maui	West Maui
AshSus-3C	kauaiensis	Kauai	Wailua River State Park
TPhy16-5A	kauaiensis	Kauai	Nualolo
AshSus-4C	kauaiensis	Kauai	Koke'e State Park
TPhy16-8H	kikokiko	Maui	Lower Waikamoi Preserve, East Maui
RGG-062_RndGldMa21GJ_62	kikokiko	Maui	Auwahi Dry Forest
RGS-023_SmSpnPWaaHA23Y11216SB2	kukuhaa	Hawaii	Pu'u Wa'awa'a
RGG-483_kukuhaa_1	kukuhaa	Hawaii	Pu'u Wa'awa'a
TPhy16-11G	kukuiki	Oahu	Pahole Natural Area Reserve
RGG-485_kukuiki_Oahu	kukuiki	Oahu	Pahole Natural Area Reserve
RGG-505_macracantha_Lanai	macracantha	Lanai	Munro Trail
TPhy16-9G	macracantha	Maui	East Maui
TPhy16-10B	macracantha	Maui	East Maui
AshSus-2A	mohihi	Kauai	Iliau Loop
AshSus-3F	mohihi	Kauai	Mohihi-Waialae
RGG-025_SLHaKa19GJ25	obscura	Hawaii	Volcanoes National Park
GutPhy-5E	obscura	Hawaii	Pu'u Maka'ala
TPhy16-1G	perreirai	Oahu	Mt. Ka'ala
TPhy16-12D	perreirai	Oahu	Mt. Ka'ala
AshSus-2F	pilosa	Kauai	Koke'e State Park
AshSus-2G	pilosa	Kauai	Koke'e State Park
TPhy16-3B	polychromata	Oahu	Mt. Ka'ala
TPhy16-12E	polychromata	Oahu	Poamoho Trail
TPhy16-8A	quasimodo	Hawaii	Alili
GutPhy-4B	quasimodo	Hawaii	Laupahoehoe
GutPhy-5H	quasimodo	Hawaii	Pu'u Maka'ala

TPhy16-2D	quasimodo	Lanai	Munro Trail
GutPhy-11G	quasimodo	Maui	Waikamoi Preserve, East Maui
TPhy16-2B	quasimodo	Molokai	Pu'u Lua
TPhy16-11H	quasimodo	Oahu	Pahole Natural Area Reserve
TPhy16-3D	restricta	Hawaii	Hakalau Forest
GutPhy-4C	restricta	Hawaii	Laupahoehoe
TPhy16-10E	restricta	Maui	East Maui
TPhy16-9A	restricta	Maui	Lower Waikamoi Preserve, East Maui
RGG-641_tantalus_2	tantalus	Oahu	Mt. Tantalus
RGG-642_tantalus_3	tantalus	Oahu	Mt. Tantalus
TPhy16-2E	waikamoi	Maui	Pu'u Kukui, West Maui
TPhy16-2F	waikamoi	Maui	Waikamoi Preserve, East Maui
GutPhy-11D	waikamoi	Maui	Waikamoi Preserve, East Maui
RGG-653_waikamoi_W._Maui_1	waikamoi	Maui	West Maui
AshSus-11H	pallescens	North America	California
TPhy16-6F	pallescens	North America	California
TPhy16-12B	"Bicolored Jaws"	Oahu	Mt. Ka'ala
TPhy16-12H	"Bicolored Jaws"	Oahu	Poamoho Trail
AshSus-3G	"Elongate Forest"	Kauai	Mohihi-Waialae
RGS-070_EFHaMKGJ70	"Elongate Forest"	Hawaii	Mauna Kea
RGG-A10_EFHaPuHuHuC5A10	"Elongate Forest"	Hawaii	Pu'u Huluhulu
RGG-008_EFKau08.JG8	"Elongate Forest"	Kauai	Waiahuakua
RGG-014_EFAuwahi00014Y40116Sb2	"Elongate Forest"	Maui	Auwahi Dry Forest
TPhy16-3H	"Emerald Ovoid"	Oahu	Mt. Ka'ala
TPhy16-4A	"Emerald Ovoid"	Oahu	Mt. Ka'ala
GutPhy-11H	"Eurylike"	Oahu	Mt. Ka'ala
RGG-038_LWOaKoo07GJ38	"Eurylike"	Oahu	Ko'olau Mountains
TPhy16-4G	"Eurylike"	Oahu	Palikea Trail
AshSus-4F	"Eurylike"	Oahu	Poamoho Trail
TPhy16-7H	"Golden Dome"	Hawaii	Alili
RGG-050_GDHiKohJG_50	"Golden Dome"	Hawaii	Kohala
GutPhy-5B	"Golden Dome"	Hawaii	Laupahoehoe
RGG-328_MenManR12	"Golden Dome"	Hawaii	Manuka
GutPhy-4F	"Golden Dome"	Hawaii	Pu'u Maka'ala
RGG-0A3_EFHaLauA3.6NZ16	"Long-Clawed Legs"	Hawaii	Laupahoehoe
TPhy16-8C	"Long-Clawed Legs"	Hawaii	Pu'u Maka'ala
RGG-323_EF.LngClKo	"Long-Clawed Legs"	Hawaii	Kohala
RGG-J33_PAOa16J33	"Pointed Abdomen"	Oahu	Ohikilolo
AshSus-3A	"Waves"	Kauai	Wailua River State Park

AshSus-2H	"Waves"	Kauai	Koke'e State Park
RGS-103_LWKaWai30GJ_103	"Waves"	Kauai	Waialeale
TPhy16-2H	acuta	Hawaii	Hualalai
RGG-047_PAHaKip14GJ47	acuta	Hawaii	Kipukas
TPhy16-3A	acuta	Hawaii	Volcanoes National Park
TPhy16-6B	acuta	Maui	Waikamoi Preserve, East Maui
GutPhy-11F	acuta	Maui	Waikamoi Preserve, East Maui
AshSus-2D	acuta	Molokai	Kamakou Preserve
TPhy16-2G	acuta	Molokai	Wailau
RGG-J65_PAMaEke.J65.6N	acuta	Maui	Mt. Eke, West Maui
RGG-J67_PAMaPKJ67.6NZ25	acuta	Maui	Pu'u Kukui, West Maui
RGG-085_PAWhMa22GJ_85	albida	Maui	Auwahi Dry Forest
RGG-S03_PAWhMa05JS3	albida	Maui	Auwahi Dry Forest
TPhy16-11C	eurychasma	Maui	East Maui
TPhy16-10C	eurychasma	Maui	East Maui
AshSus-2E	eurychasma	Molokai	Kamakou Preserve
AshSus-4E	eurychasma	Molokai	Kamakou Preserve
RGG-281_LWMAPk1	eurychasma	Maui	Pu'u Kukui, West Maui
RGS-061_LW.WM20GJ_61	eurychasma	Maui	Pu'u Kukui, West Maui
TPhy16-10A	filiciphilia	Maui	East Maui
AshSus-2B	filiciphilia	Molokai	Kamakou Preserve
AshSus-2C	filiciphilia	Molokai	Kamakou Preserve
RGS-020_filMa04JG20	filiciphilia	Maui	Waikamoi Preserve, East Maui
GutPhy-3H	hawaiensis	Hawaii	Pu'u Maka'ala
AshSus-3D	hawaiensis	Kauai	Wailua River State Park
TPhy16-3F	hawaiensis	Kauai	Nualolo
TPhy16-5C	hawaiensis	Oahu	Mt. Ka'ala
RGG-0A8_MSMauiA8.6NZ35	hawaiensis	Maui	Kipahulu, East Maui
RGG-067_PAHawai0006716Sb2Y659041001	kea	Hawaii	Hakalau Forest
RGG-051_PAWhHa24GJ51	kea	Hawaii	Pu'u Wa'awa'a
RGG-A27_PAWhtGhoA3A27	kea	Hawaii	Hakalau Forest
TPhy16-4B	lena	Oahu	Mt. Tantalus
TPhy16-4C	lena	Oahu	Mt. Tantalus
RGG-J26_OaK33J26	limu	Oahu	Mt. Ka'ala
RGG-B16_OaKonahC3A1	limu	Oahu	Konahuanui
RGG-066_PAKaua0006616Sb2Y657041001	maka	Kauai	Koke'e State Park
AshSus-4A	maka	Kauai	Koke'e State Park
RGG-339_OPal31.N	palikea	Oahu	Palikea Trail
RGG-349_OPoa8.N	palikea	Oahu	Poamoho Trail
TPhy16-10H	paludicola	Maui	East Maui
AshSus-4D	paludicola	Molokai	Kamakou Preserve

RGG-J54_RndGrnMo35J54	paludicola	Molokai	Pu'u Lua
RGG-J55_RndGrnMaK34J55	paludicola	Maui	Kipahulu, East Maui
RGG-291_RGMaPKHC3	paludicola	Maui	Pu'u Kukui, West Maui
TPhy16-7G	perkinsi	Hawaii	Alili
GutPhy-5D	perkinsi	Hawaii	Pu'u Maka'ala
GutPhy-4E	redstar	Hawaii	Pu'u Maka'ala
TPhy16-8F	RedStar	Hawaii	Volcanoes National Park
TPhy16-11E	stelarobusta	Maui	East Maui
TPhy16-4D	stelarobusta	Maui	Pu'u Kukui, West Maui
TPhy16-4E	stelarobusta	Maui	Pu'u Kukui, West Maui
RGG-J50_EFMaFl18J50	stelarobusta	Maui	Waikamoi Preserve, East Maui
TPhy16-6A	trituberculata	Maui	Waikamoi Preserve, East Maui
GutPhy-11A	trituberculata	Maui	Waikamoi Preserve, East Maui
RGS-J71_OaHa09J71	uluhe	Oahu	Halona Valley
AshSus-12D	versicolor	North America	California
TPhy16-6E	versicolor	North America	California
TPhy16-1B	viridis	North America	Maine