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Multiple paths toward repeated phenotypic evolution in the spiny-leg adaptive radiation (*Tetragnatha*; Hawai'i)

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Abstract

The repeated evolution of phenotypes provides clear evidence for the role of natural selection in driving evolutionary change. However, the evolutionary origin of repeated phenotypes can be difficult to disentangle as it can arise from a combination of factors such as gene flow, shared ancestral polymorphisms or mutation. Here, we investigate the presence of these evolutionary processes in the Hawaiian spiny-leg *Tetragnatha* adaptive radiation, which includes four microhabitat-specialists or ecomorphs, with different body pigmentation and size (Green, Large Brown, Maroon, and Small Brown). We investigated the evolutionary history of this radiation using 76 newly generated low-coverage, whole-genome resequenced samples, along with phylogenetic and population genomic tools. Considering the Green ecomorph as the ancestral state, our results suggest that the Green ecomorph likely re-evolved once, the Large Brown and Maroon ecomorphs evolved twice and the Small Brown evolved three times. We found that the evolution of the Maroon and Small Brown ecomorphs likely involved ancestral hybridization events, while the Green and Large Brown ecomorphs likely

evolved through novel mutations, despite a high rate of incomplete lineage sorting in the dataset. Our findings demonstrate that the repeated evolution of ecomorphs in the Hawaiian spiny-leg *Tetragnatha* is influenced by multiple evolutionary processes.

KEYWORDS
ancestral shared polymorphism, Araneae, convergent evolution, de novo mutation, genomic divergence, hybridization, introgression, parallel evolution, standing genetic variation, Tetragnathidae

1 | INTRODUCTION

Adaptive radiation is an evolutionary process in which a single ancestral lineage diversifies into multiple phenotypically distinct species adapted to different ecological niches. This process offers an excellent opportunity to investigate the links between phenotypic diversification and environmental adaptation (Gillespie et al., 2020; Schluter, 2000), as well as the genomic basis mediating evolutionary and ecological change (Cerca et al., 2023; De-Kayne et al., 2022; Marques et al., 2022).

The repeated evolution of phenotypes, commonly referred to as parallel or convergent evolution (Cerca, 2022; James et al., 2023), is a particularly fascinating aspect of adaptive radiations as it provides valuable insights into the extent to which phenotypic outcomes in response to similar environmental conditions are predictable (Gillespie et al., 2018, 2020; Losos, 2010, 2011; Losos & Ricklefs, 2009; Malinsky et al., 2018; Masonick et al., 2022; Salzburger, 2018; Urban et al., 2022). For example, the repeated evolution of habitat specialists has been documented in multiple adaptive radiations including the Caribbean *Anolis* lizards (Losos & Ricklefs, 2009), Hawaiian *Tetragnatha* (Gillespie, 2004), cichlid fishes (Sowersby et al., 2021) and *Ariamnes* spiders (Gillespie et al., 2018). In most of these cases, the spatial segregation of environments has been proposed as an ecological basis facilitating repeated evolution (Losos, 2010; Losos & Ricklefs, 2009). Nonetheless, our understanding of the evolutionary processes contributing to the repeated emergence of phenotypes remains incomplete.

The Hawaiian *Tetragnatha* spiny-leg radiation consists of approximately 17 species and is part of a larger radiation endemic to the archipelago (Gillespie, 2016; Kennedy et al., 2022). The spiny-legs can be categorized into four groups of microhabitat specialists based on the substrate they inhabit and their phenotypic traits (Gillespie, 2004): the Large Brown is found on tree bark (Figure 1a), the Green on leaves (Figure 1b), the Maroon on mosses (Figure 1c) and the Small Brown on twigs (Figure 1d). These groups have been classified as ecomorphs, a term originally defined by Ernest E. Williams as "species with the same structural habitat/niche, similar in morphology and behavior, but not necessarily close phyletically" (Williams, 1972). The spiny-leg radiation is considered to have colonized the Hawaiian archipelago around 5.1 million years ago, at the time of the emergence of the oldest high-elevation island of Kaua'i (Kennedy et al., 2022), and ancestral character-state reconstructions suggest that the Green ecomorph is likely the ancestral form (Gillespie, 2004). Recent genomic

studies have shown that co-occurring closely related species within the Green ecomorph do not hybridize (Cotoras et al., 2018), and it has been proposed that the evolution of ecomorphs may occur through cycles of allopatry, secondary contact, and subsequent macroevolutionary character displacement driven by directional selection (Cotoras et al., 2018; Schluter, 2000).

The repeated evolution of phenotypes or ecomorphs can result from three evolutionary processes that are not mutually exclusive and that can occur at different regions of the genome (Cerca, 2022; Lee & Coop, 2017, 2019; Pease et al., 2016; Stern, 2013): mutation (either *de novo* or larger mutational events, where different mutations cause similar phenotypes), shared ancestral polymorphism (standing variation, where old genetic variation is recruited) or gene flow (where an allele is recruited from one lineage to another). These three patterns have been observed in the context of adaptive radiations (Choi et al., 2021; De-Kayne et al., 2022; Meier et al., 2018; Richards & Martin, 2017; Salzburger, 2018; Sowersby et al., 2021) and, because they leave distinct footprints along the genome, the use of genomic-level data makes it possible to distinguish the contribution of each of these processes (Barrett & Schluter, 2008; Lee & Coop, 2017, 2019).

Here, we performed whole-genome re-sequencing of 76 genomes across the *Tetragnatha* spiny-leg radiation with the aim of understanding the presence of the three aforementioned evolutionary processes that can underlie repeated ecomorph evolution. We start by reconstructing the evolutionary history of the spiny-leg lineage using phylogenetic tools. Then, we explore patterns of excess allele sharing to infer potential hybridization, study the relationship between genomic divergence (D_{XY}) and excess allele sharing, and uncover the contribution of mutation versus standing genetic variation through the use of phylogenies for different genomic regions. Our results uncover a complex evolutionary history, showing that the repeated evolution of ecomorphs may have emerged through multiple different evolutionary processes.

2 | METHODS

2.1 | Field collection

Spiny-leg *Tetragnatha* spiders inhabit the montane rain forests located on all major Hawaiian islands, at elevations ranging from 1200 to 1800 m (Roderick et al., 2012). To collect specimens, spiders were either manually collected during their active hours at night or using a

FIGURE 1 *Tetragnatha* spiny-leg ecomorphs and study area. (a) Large Brown (*Tetragnatha quasimodo*; represented by a brown diamond), (b) Green (*Tetragnatha brevignatha*; represented by a green triangle), (c) Maroon (*Tetragnatha kamakou*; represented by a maroon star) and (d) Small Brown (*Tetragnatha anuenue*; represented by a brown square). (e) Map of Hawai'i coloured by elevation, with an indication of sampling regions and species. Photographs by Darko D. Cotoras.
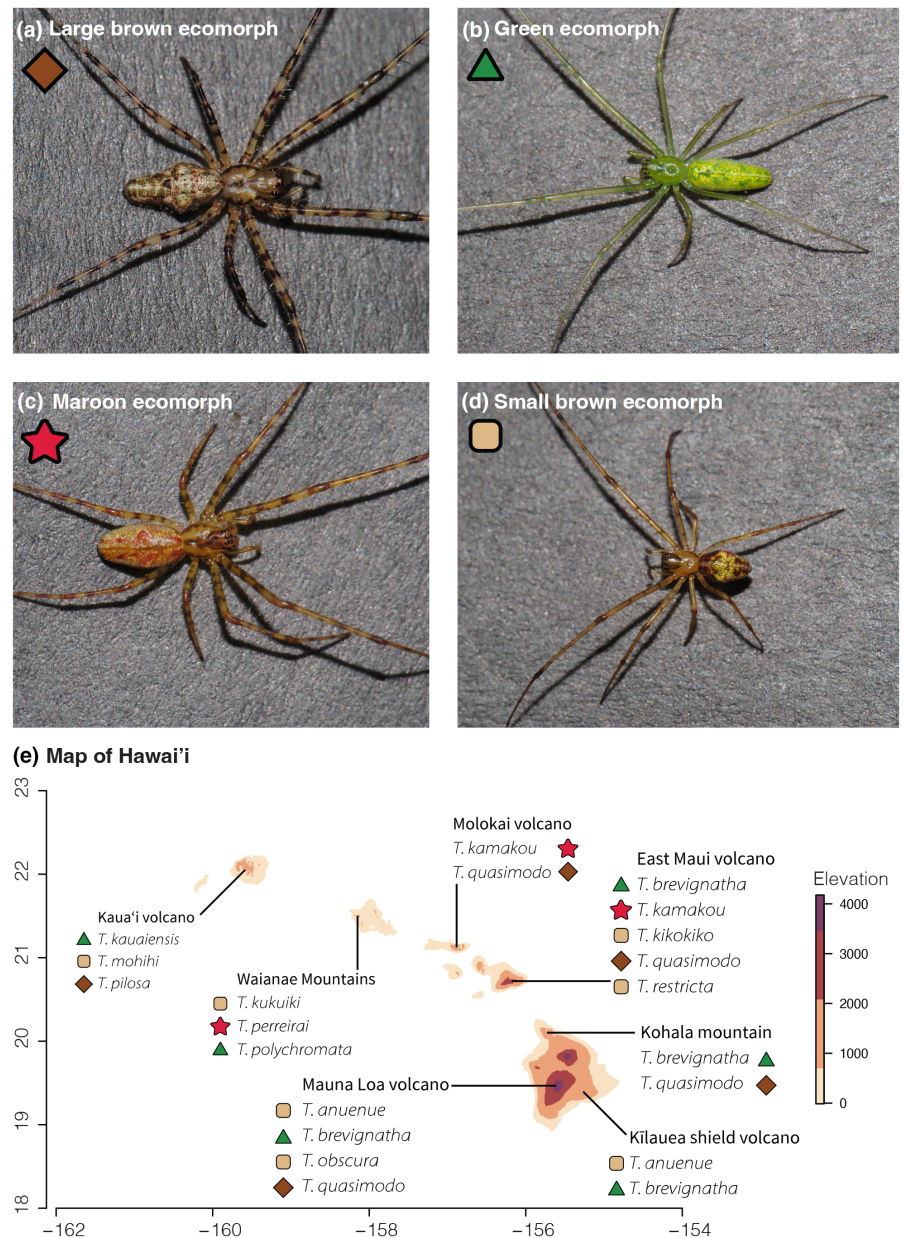
beating sheet during both day and night. After collection, specimens were preserved in 95% ethanol and stored at −20°C. A complete list of specimens, along with their collection site coordinates (volcano and island), is provided in Table S1.

2.2 | Molecular data generation

We generated genome sequences from 76 spiny-leg *Tetragnatha* individuals (Table S1). DNA was extracted from 2 to 4 legs of each specimen by grinding the legs with a tube pestle and incubating them overnight in a lysis buffer solution (10 mM Tris pH, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) and proteinase K at 56°C. We used a commercial provider kit, Qiagen, to extract DNA, eluted the DNA in 50 μL of elution buffer, and assessed the concentration of each DNA extract using a Qubit fluorometer (ThermoFisher). Samples with more than 500 ng of DNA were submitted to the QB3-Berkeley, Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley for library preparation.

The DNA was fragmented using a Bioruptor Pico (Diagenode), and libraries were prepared using the KAPA Hyper Prep kit for DNA (KK8504). Truncated universal stub adapters were added to the DNA-adapter ligation, and indexed primers were used for PCR amplification to complete the adapters. The quality of the samples was checked on an AATI Fragment Analyser (now Agilent), and the molarity of the library was measured using quantitative PCR with the KAPA Library Quantification Kit (Roche KK4824) on a BioRad CFX Connect thermal cycler. Libraries were pooled by molarity and sequenced on an Illumina NovaSeq 6000 S4 flowcell for 2 × 150 cycles, targeting 10 Gbp per sample. Raw sequencing data was converted to FASTQ format and demultiplexed using Illumina's bcl2fastq2 software, allowing for up to one mismatch in the index sequences.

2.3 | Bioinformatics data processing

All the bioinformatic processing steps reported below are available in https://github.com/jcerca/Papers/tree/main/Tetragnatha_MolEcol. The quality of the sequencing data was checked using fastQC v0.11.8 (Andrews, 2017), and adapters were identified using AdapterRemoval v2.3.1 (--identify-adapters; Schubert et al., 2016). Trimmomatic v0.39 (Bolger et al., 2014) was then used to remove adapters and poor-quality reads by specifying: maximum of two mismatches in the adapter sequence; to cut three base pairs in the beginning and the end of the read if the quality drops below 20 in these regions; a sliding window of 4 bp with a minimum quality threshold of 20; and a minimum read length of 50. The high-quality reads were aligned to the *Tetragnatha kauaiensis* reference genome v1 (~1.1 Gb; Cerca et al., 2021) using the Burrows-Wheeler Aligner v0.7.17, mem algorithm (Li & Durbin, 2009). All four files from Trimmomatic (forward-paired, reverse-paired, forward-unpaired, and reverse-unpaired) were aligned to the reference genome, merged and sorted using Samtools v1.10 (Li et al., 2009) to avoid data loss as done in Ravinet et al. (2018). The mapping quality of each alignment was estimated using samtools flagstat, which showed no mapping bias (Table S1), and duplicates were marked using GATK 4.1.4.0 (McKenna et al., 2010), using the SortSam algorithm to sort reads based on genomic coordinates, followed by the MarkDuplicates function. Indexes for each alignment were built using BuildBamIndex (McKenna et al., 2010), reads with mapping quality below 30 were discarded, and sequencing depth was calculated using samtools depth (option -a was used to output all sequencing positions, even those with 0 depth; Table S1). Finally, as our data ranged between 2.24 and 8.22× (Table S1), we processed it using ANGSD v0.935 (Kousathanas et al., 2017), a pipeline designed to handle and analyse low-coverage sequencing data.

2.4 | Phylogenetic analyses

We began our analyses by reconstructing the evolutionary history of the radiation using phylogenetic tools. To achieve this, we included specimens from an outgroup lineage, the *Tetragnatha* web builder radiation, which is also endemic to Hawai'i (*T. maka*, *T. acuta*, *T. filiciphilia*, and *T. stelarobusta*). We called variants in ANGSD using the GATK genotype likelihood model to output a beagle file (-GL 2 -doGlf 2), while specifying that a variant should be present in at least 38 individuals (half of the dataset), a minimum base quality of 30 (-minQ), and a *p*-value threshold of 1e-6 (-SNP_pval). We also removed allele counts in less than 5% of the dataset (-minMaf 0.05) and inferred major and minor alleles from the genotype likelihoods (-doMajorMinor 1). Next, we removed variants in repeat regions using an in-house script (see Github). We then conducted various phylogenetic reconstructions:

1. We used SplitsTree v4 to create a phylogenetic network (Huson & Bryant, 2005). Due to the large number of sites obtained, we ran the analysis on a reduced dataset of 100,000 randomly selected variants from the genome, including only variants with coverage between 7 and 30 (genotype likelihood depth filter between 7 and 30).

2. We used NgsDist (Vieira et al., 2015), a software optimized to run trees on whole-genome low-coverage data to determine whole-genome signals. We obtained a phylogenetic representation of the whole genome, specifying a block size of 20 SNPs, and 100 bootstrap replicates (1,848,915 sites). We then ran the NJ software fastme v2 (Lefort et al., 2015) and merged all 100 replicates into a tree with bootstrap support using RAXML 8.2.12 (Stamatakis, 2014) while specifying an optimization of branch length and GTRCAT (GTR+Optimization of substitution rates+Optimization of site-specific) as the substitution model. Because low-coverage methods rely on neighbour-joining approaches, we ran IQ-TREE v2.0.3 (Minh et al., 2020) on a set of ultra-conserved elements extracted with phyluce (Faircloth, 2016; Faircloth et al., 2012; Starrett et al., 2017) with the GTR model and 1000 ultrafast bootstraps to compare topologies.

3. In order to investigate the possibility of nuclear-mitochondrial discordances, we performed a phylogenetic reconstruction of mitochondrial genomes. We extracted the mitochondrial genomes from the cleaned Illumina sequences using Novoplasty v4.2 (Dierckxsens et al., 2017). We obtained a total of 46 fully-circularized mitochondrial genomes and 19 partially complete mitochondrial genomes (>5000 bp). We then concatenated and aligned the 65 mitogenomes using Mafft v7.130b (Katoh & Standley, 2013), trimmed the ends of the alignments, and obtained a tree by running IQ-Tree v2.0.3 with 1000 ultrafast bootstrap replicates and automatic determination of substitution models.

2.5 | Hybridization and partitioning of genetic variation

We investigated whether hybridization (interspecific gene flow) played a role in the evolution of the spiny-leg radiation by examining patterns of allele sharing. For this, we applied a dataset filter between 7 and 30× coverage, as used for the SplitsTree analysis, and used Dsuite (Malinsky et al., 2021) to calculate Patterson's D (ABBA-BABA) and F4 ratios based on nuclear markers (Patterson et al., 2012). Because Dsuite offers a convenient way to study all possible Patterson's D and F4 topologies, we used this tool to investigate the extent of excess allele sharing between species belonging to the same ecomorph and between species belonging to different ecomorphs.

Because we found evidence of excess allele sharing between species belonging to the same ecomorph, particularly in the maroon and small brown ecomorphs, we conducted a follow-up investigation focusing on determining the relationship between excess allele sharing (F_{dM}—a method which has been implemented and optimized for *F*-statistic analyses of genomic windows; Malinsky et al., 2015) and genomic divergence (D_{XY}). We reasoned

that regions with high F_{dM} and high D_{XY} are indicative of ancestral hybridization events. In these cases, a particular region may have experienced introgression, leading to an initial reduction in D_{XY}. However, over time, there is the accumulation of divergence (an increase of D_{XY}) along with excess allele sharing (high F_{dM}). Regions with high F_{dM} and low D_{XY} likely indicate more recent hybridization as divergence has not yet accumulated. However, because some genomic regions may accumulate divergence by chance (e.g. elevated mutation rates), we explored the relationship between F_{dM} and D_{XY} using different pairs of species. Specifically, we explored D_{XY}-F_{dM} associations between species belonging to the same ecomorph (e.g. Maroon 1 vs. Maroon 2), and extended these associations to a third species belonging to another ecomorph (e.g. Maroon 1 vs. Green 1, Maroon 2 vs. Green 1). We used Dsuite's investigate algorithm to obtain F_{dM} ratios using windows of 50 SNPs with a 25 SNP step size, and 'genomicsgeneral' by Simon Martin to obtain D_{XY} estimates (Martin & Jiggins, 2017; Martin & Van Belleghem, 2017). We extracted the top 1% regions with positive F_{dM} (i.e. highest F_{dM} values), keeping only positive values (i.e. those concerning the P2-P3 comparison, which was of interest), and compared D_{XY} values from these windows to other regions of the genome, subsetting the genome to a random 1%. To test for differences between means of the top 1% and random 1% of the genome we performed the Wilcoxon test with the rstatix package (Kassambara, 2020).

To further investigate the possible effects of hybridization on population structure, we performed a NGSAdmix analysis using ANGSD (Skotte et al., 2013). We applied the same filters as for the phylogenetic reconstruction with NgsDist, with the exceptions of a minimum base quality of 20 and a minimum allele frequency of 0.05. Additionally, we calculated linkage disequilibrium in windows of 100,000 bps as done in (Sowersby et al., 2021), and used plink to prune the data by removing genomic regions where linkage, measured as r^2, was above 0.11 in windows of 25,000 bps. We ran NGSadmix analyses with cluster values (K) ranging from 1 to 16 (total number of species herein included, 15, +1) and found that the best K value was 2 according to the Evanno method as implemented in CLUMPAK (Evanno et al., 2005). We presented the results for $K = 2$, 3, and 15 as ADMIXTURE plots to examine how genetic variation is partitioned at different levels (Meirmans, 2015).

2.6 | The contribution of mutation and standing genetic variation

To distinguish between the role of standing genetic variation and mutations in driving phenotypic evolution in the Green and Large Brown ecomorphs, the two ecomorphs without evidence of within-ecomorph excess allele sharing, we employed the following reasoning: If standing genetic variation underlies ecomorph evolution, certain genomic regions should group all members of a given ecomorph together as monophyletic, since selection acted on common genetic variation. If mutations underlie the evolution of ecomorphs, we expect all members of a given ecomorph to be polyphyletic, as they represent distinct evolutionary origins. Note that we assume that it is unlikely that the same mutations will evolve independently in separate species, although it is possible. To test this, we split the genome into blocks spanning 20,000 bps and selected those with at least 100 SNPs, obtaining a total of 4275 regions. For each of these regions, we did phylogenetic reconstructions using IQ-TREE (saving ultrafast bootstrap trees without branch lengths), and used phykit monophyly_check (Steenwyk et al., 2021) to determine if there were any regions of the genome where either all Green or all Large Brown species were monophyletic.

Finally, to evaluate the contribution of incomplete lineage sorting, a process which is strongly linked to standing genetic variation and quite common in fast radiations such as the spiny-legs, we calculated gene concordance factors (gCF; proportion of genes supporting the species tree) and site concordance factors (sCF; proportion of sites supporting the species tree) using IQ-TREE. We then ran ASTRAL v5.7.8 to estimate the species tree under the multispecies coalescent (Mirarab et al., 2014; Rabiee & Mirarab, 2020). ASTRAL is designed for inferring species trees from collections of gene trees, addressing issues of tree incongruence, and is thus better suited to understanding incomplete lineage sorting. To run ASTRAL we used two input files: the collection of bootstrapped trees (to estimate node support on the species trees) and the maximum-likelihood trees for each of the 4275 regions.

3 | RESULTS

3.1 | Tree reconstruction

The phylogenetic network shows three major groups (Figure 2a). The first group includes the spiny-leg lineages from Kaua'i, namely *Tetragnatha pilosa* (Large Brown), *T. kauaiensis* (Green), and *T. mohihi* (Small Brown). Notably, one individual of *T. mohihi* is nested together with *T. kauaiensis*. The second group includes *T. quasimodo* (Large Brown), and a cluster of Small Brown species, including *T. anuenue*, *T. kikokiko*, *T. kukuiki* and *T. obscura*. The third group includes several species of the Green ecomorph (*T. polychromata*, *T. brevignatha*, *T. tantalus*, *T. waikamoi*), two maroon species (*T. perreirai* and *T. kamakou*) and one Small Brown species (*T. restricta*; Figure 2a).

In the nuclear tree (Figure 2b), *T. pilosa* is sister to all other spiny-leg species, but the node including all other spiny-legs has a bootstrap support below 95, being the only interspecific node with a bootstrap support below 100. Notably, the node including all spiny-legs except *T. pilosa* is preceded by a very short branch and separates two clades: one clade including *T. mohihi* and *T. kauaiensis*, and the other clade including all remaining species. Hereafter, we consider *T. pilosa*, *T. mohihi* and *T. kauaiensis* as part of the clade A (recovered as monophyletic in the mitochondrial tree). The remaining species can be separated into two major clades: the first, clade B, includes
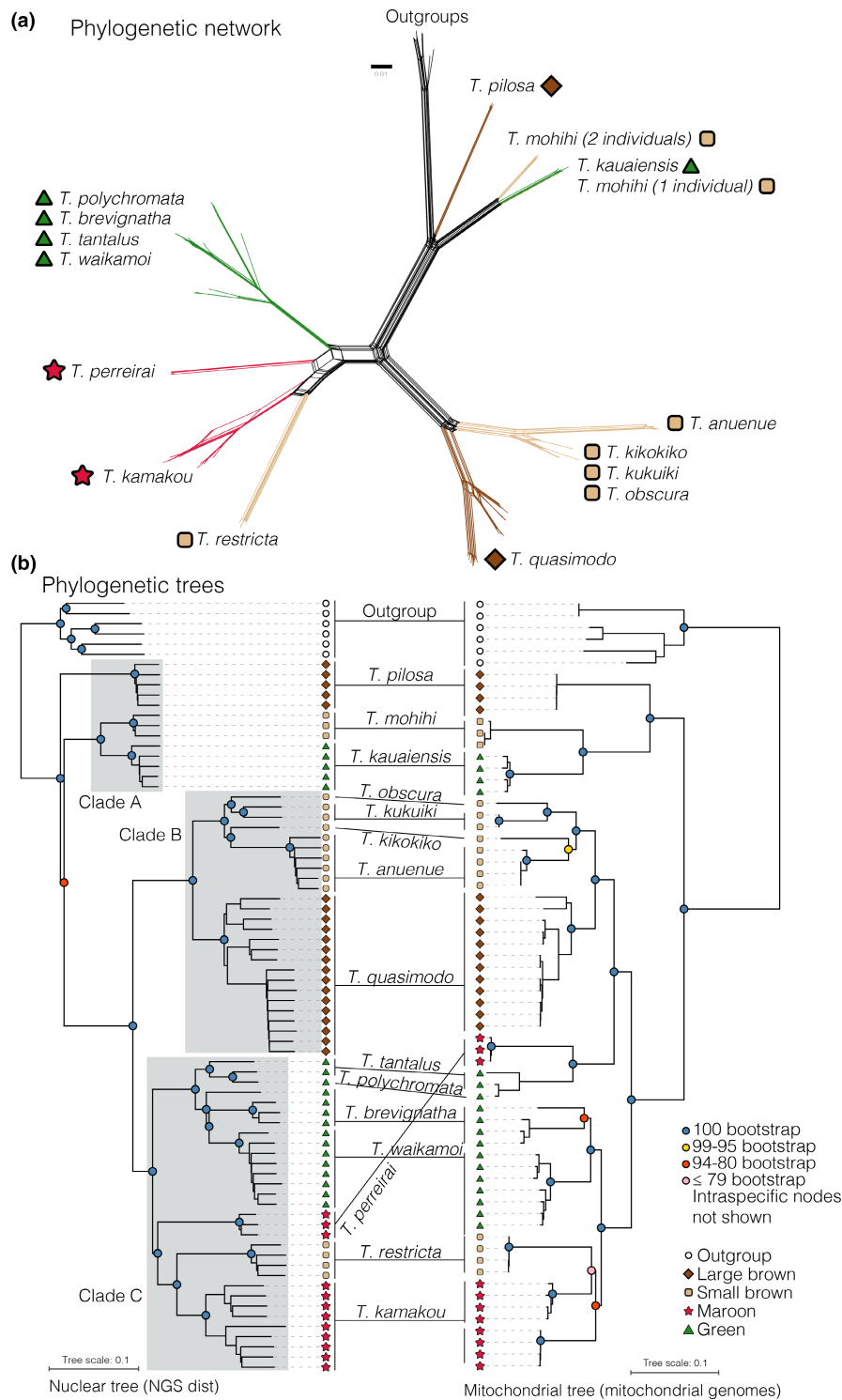
FIGURE 2 Phylogenetic reconstructions. (a) SplitsTree network based on 100,000 polymorphisms with species and ecomorph label (the Large Brown ecomorph is represented by a brown diamond, the Green ecomorph by a green triangle, the Maroon ecomorph by a maroon star, and the Small Brown ecomorph by a brown square. (b) Phylogenetic tree reconstruction for the nuclear genome (left) obtained by NgsDist is based on 1,848,915 variable sites. The mitochondrial tree (right) obtained by NovoPlasty and IQ-Tree is based on complete and partial mitochondrial genomes. Bootstrap is provided for each interspecific node, and the tips of the phylogeny include ecomorph and outgroup labelling as coloured symbols. Clades A–C are plotted in the nuclear tree.

the Large Brown *T. quasimodo* and a group of Small Brown species, including *T. obscura, T. kukuiki, T. kikokiko* and *T. anuenue*; and the second, Clade C, includes a group of species representing the Green

ecomorph (*T. tantalus, T. polychromata, T. waikamoi, T. brevignatha*), which is sister to a clade comprising the two Maroon species (*T. perreirai* and *T. kamakou*) and a Small Brown species (*T. restricta*). Similar

FIGURE 3 Tree topology obtained by ASTRAL, annotated with gene concordance factors and site concordance factors. All branches have a posterior probability of 1 except for one branch, marked with an asterisk, which has a posterior probability of 0.67.
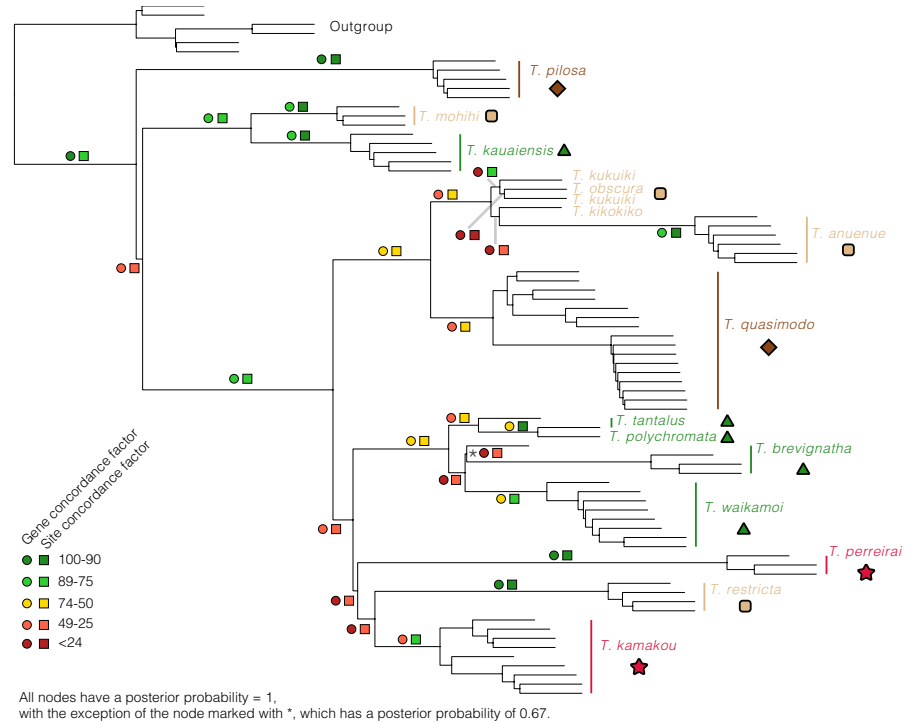
to the network reconstruction, the Maroon ecomorph is recovered as polyphyletic. The ASTRAL coalescent-based reconstruction is topologically consistent with the NgsDist tree (Figures 2 and 3) and the UCE-based tree (Figure S1).

There are some discordances between the mitochondrial and nuclear trees, as shown by differences in topology and bootstrap support (Figure 2b). First, in the mitochondrial tree, *T. pilosa* groups together with *T. mohihi* and *T. kauaiensis*, forming a monophyletic cluster that is not observed in the nuclear tree. In contrast to the nuclear tree, the basal nodes in the mitochondrial tree have a bootstrap support of 100, and lower bootstrap notes are found when separating some of the sister species. Second, in the mitochondrial tree, *T. tantalus* and *T. polychromata* (Green ecomorphs) are sister to *T. perreirai* (Maroon ecomorph), and this clade is sister to the clade including the Large Brown (*T. quasimodo*) and Small Brown ecomorphs (*T. anuenue*, *T. kikokiko*, *T. kukuiki*, *T. obscura*), with 100% bootstrap support at the interspecific nodes. In contrast to the mitochondrial data, where three Green clades occur, the nuclear tree suggests two clades of Green ecomorphs (1—*T. kauaiensis*; 2—*T. tantalus*, *T. polychromata*, *T. waikamoi* and *T. brevignatha*). Third, the mitochondrial dataset suggests *T. kamakou* is paraphyletic, separating the populations from Moloka'i and East Maui volcano but this node has weak bootstrap support (below 80).

3.2 | Excess allele sharing

We observed excess allele sharing within and between ecomorphs, as indicated by Patterson's *D* (Table S2, Figure S2) and F4-branch statistics (Figure 4). The two Maroon ecomorph species (*T. perreirai*

and *T. kamakou*) showed the highest level of excess allele sharing (trio P1 = *T. restricta*; P2 = *T. kamakou*; P3 = *T. perreirai* had a *D*-statistic of 0.15, *Z*-score 30.42; F4-ratio of 0.102 over >150,000 sites). Additionally, we found evidence of excess allele sharing between Small Brown lineages, specifically between the lineages *T. restricta* (*Small Brown*)/kamakou (*Maroon*) and the other Small Brown clade (e.g. trio P1 = *T. perreirai*; P2 = *T. restricta*; P3 = *T. anuenue*, *T. kukuiki*, *T. kikokiko*, *T. obscura* trio, with a *D*-statistic of 0.13, *Z*-score of 8.21; F4-ratio of 0.07, over >150,000 sites). We did not detect high excess allele sharing in the Green and Large Brown ecomorphs (Figure 4).

When comparing between species belonging to different ecomorphs, we found evidence of excess allele sharing between the lineages of clade A, namely between *T. pilosa* (Large Brown) and *T. kauaiensis* (Green; e.g. Trio P1 = *T. kamakou*; P2 = *T. kauaiensis*; P3 = *T. pilosa*, with a *D*-statistic of 0.08, *Z*-score of 21.25, and a F4-ratio of 0.05; over 120,000 sites) and *T. mohihi* (Small Brown; e.g. Trio P1 = *T. kamakou*; P2 = *T. mohihi*; P3 = *T. pilosa*, with a *D*-statistic of 0.09, *Z*-score of 16.37, and a F4-ratio of 0.052; around ~100,000 sites). We also observed an excess of allele sharing between the Green ecomorph group (*T. tantalus*, *T. polychromata*, *T. brevignatha*, *T. waikamoi*) and one of the Maroon ecomorph species (*T. kamakou*; e.g. Trio P1 = *T. restricta*; P2 = *T. kamakou*; P3 = *T. tantalus*, *T. polychromata*, *T. brevignatha*, *T. waikamoi*, with a *D*-statistic of 0.1, *Z*-score of 11.25, and a F4-ratio of 0.088; over 150,000 sites). *T. quasimodo*, one of the Large Brown species, had excess allele sharing with the clade consisting of *T. restricta* (Small Brown; e.g. Trio P1 = *T. perreirai*; P2 = *T. restricta*; P3 = *T. quasimodo*, with a *D*-statistic of 0.12, *Z*-score of 8.12, and a F4-ratio of 0.076; over 150,000 sites) and *T. kamakou* (Maroon; e.g. Trio P1 = *T. perreirai*; P2 = *T. kamakou*; P3 = *quasimodo*, with a *D*-statistic of 0.13, *Z*-score of 9, and a F4-ratio of 0.077; over 180,000 sites).
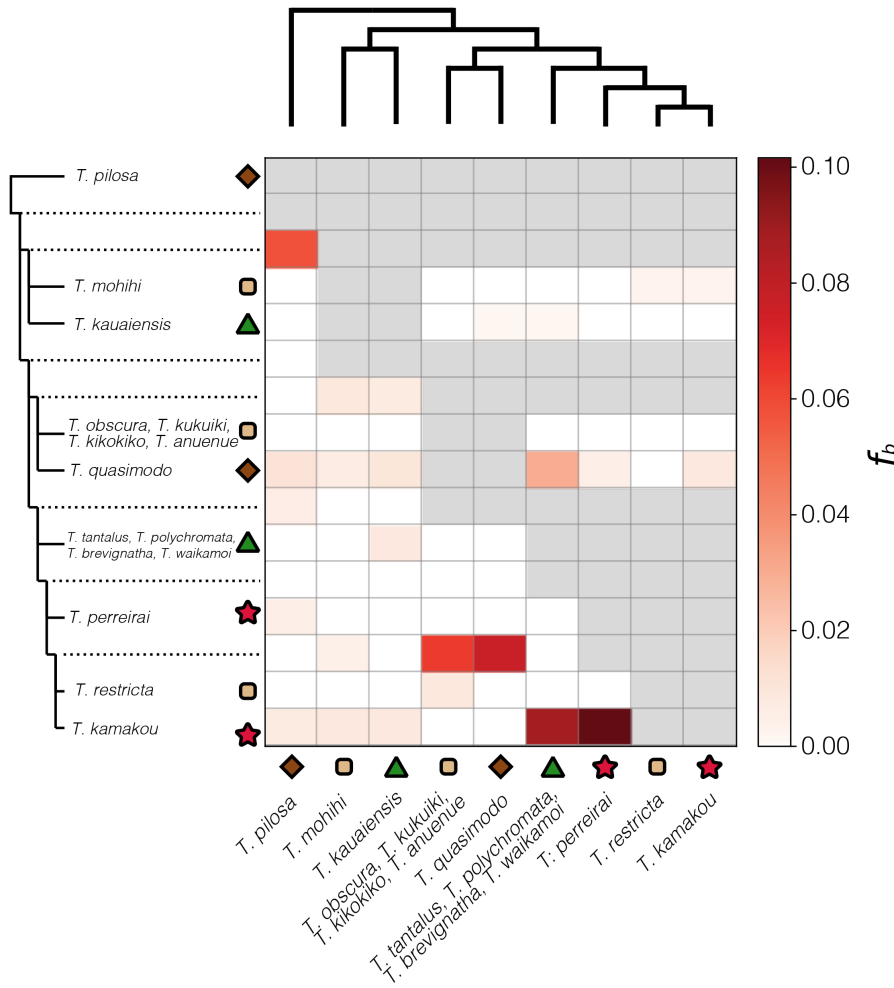
FIGURE 4 Excess allele sharing. F4-branch statistic plotted as a heatmap. The tree topology obtained by NgsDist is plotted above, and on the left, every branch of the tree is displayed (including internal branches). Because the goal is to understand hybridization between ecomorphs, clades of closely related species belonging to the same ecomorph (i.e. monophyletic ecomorphs) are displayed. Grey boxes represent comparisons that could not be established due to the topological constraints of the F4 test.

3.3 | Structuring of genetic diversity

The Evanno method suggested $K=2$ as the best K, which separated clade B from the remaining clades established in the phylogenetic reconstructions (A and C; Figure 5). $K=3$ separated all three clades, indicating evidence of shared history for both K values, with some species having minor components of other clades. In addition to displaying $K=2$ and $K=3$, we also show $K=15$, which is the number of species in the dataset. At $K=15$, some species were assigned to the same genetic cluster, such as *T. kauaiensis* and *T. mohihi* (red), *T. obscura* and *T. kukuiki* (green), and *T. polychromata* and *T. tantalus* (aqua). Interestingly, both Large Brown species, *T. pilosa* and *T. quasimodo*, showed intraspecific population structure, with *T. pilosa* having two genetic clusters (silver and blue) and *T. quasimodo* having three (cyan, orange-red and golden). Finally, only one *T. brevignatha* sample had mixed ancestries, all in common with species belonging to the same cluster of Green ecomorphs.

We obtained estimates of D_{XY} for regions with different levels of F_{dM}, establishing a link between genomic divergence and hybridization. We expected that recent introgression leads to a sharp decline of genomic divergence (D_{XY}) in regions of the genome where excess allele sharing is found (high F_{dM}). On the other

hand, ancestral hybridization would have a less sharp decrease of divergence (D_{XY}) in regions of excess allele sharing (high F_{dM}). In every comparison, whole genome D_{XY} estimates (green) were higher than the estimates of the top 1% regions with high F_{dM} (orange; Figure 6). Only one within-ecomorph comparison was not significant (Figure 6b). The regions with high F_{dM} in same-ecotype comparisons did not show any skew when including another ecomorph (pink—light green; yellow—brown), with the exception of the *T. anuenue*—*T. kauaiensis* comparison, where we find a significantly higher D_{XY} in the regions where *T. anuenue* and *T. restricta* have high F_{dM}. Comparisons between the top 1% and lowest 1% of F_{dM} yielded similar results to the comparison between the top 1% and the genomic background (Figure S3).

3.4 | ILS and de novo mutation

The ASTRAL analysis showed a posterior probability of 1 for every interspecific node, with the exception of the *T. brevignatha* node (Figure 3; Figure S4). However, the gene and site concordance analyses provided a different perspective. Specifically, with the exception of four branches (the branch separating the outgroup and the spiny-leg radiation, the *T. restricta* branch, the *T. perreirai* branch, and the
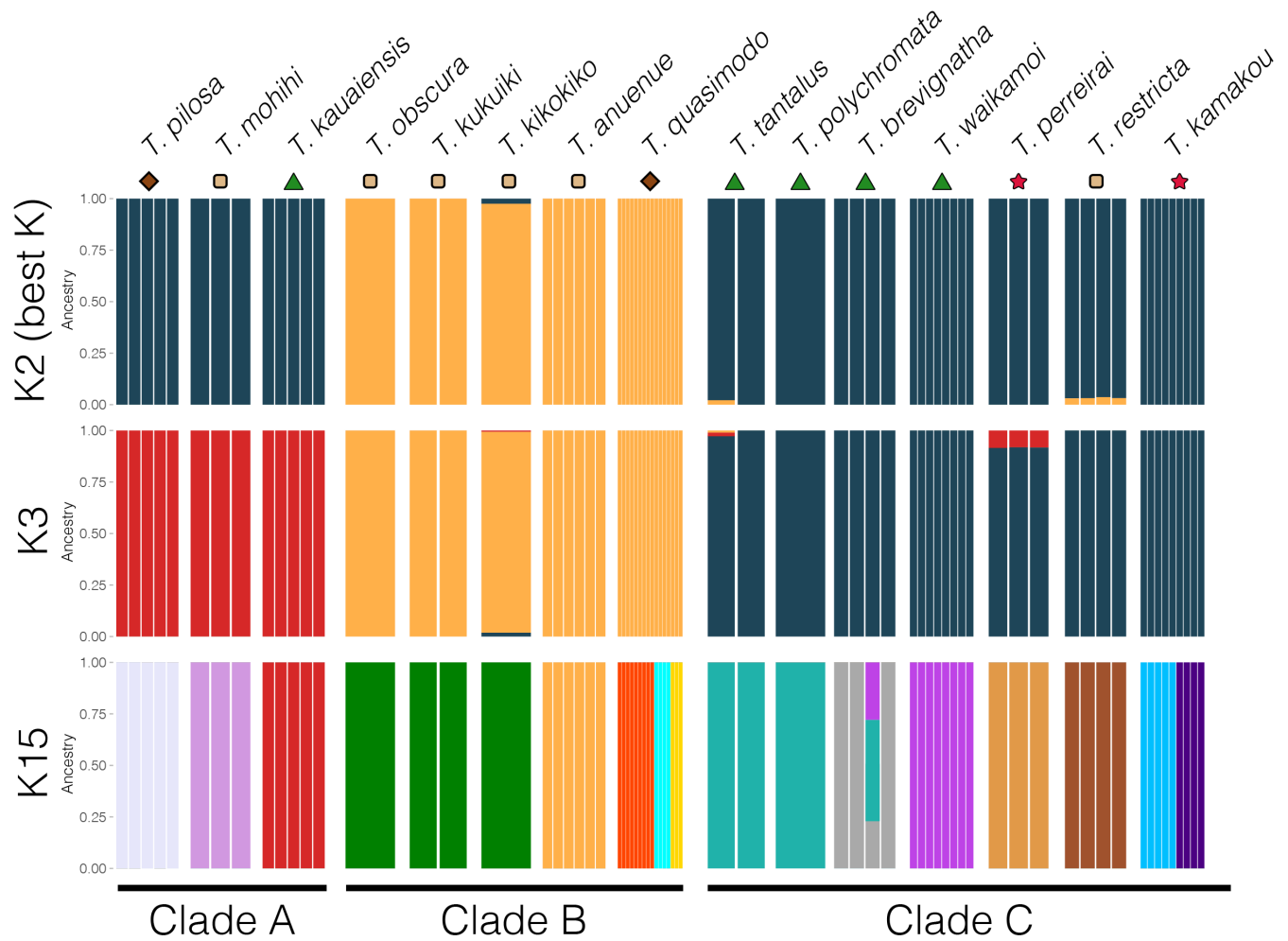
FIGURE 5 Structure of genetic diversity. NgsAdmix analysis with cluster (*K*) values of 2 (best *K*), 3 (major clades) and 15 (number of species).

branch for *T. kauaiensis*), gene concordance factors were all below 90, and 7 out of 25 branches had gene concordance factors below 30. Similar to gene concordance factors, site concordance factors were also low.

We obtained 4275 genomic regions of 20,000bps with more than 100 SNPs, and, for each of these regions, we constructed phylogenetic trees to explore whether the Green or Large Brown ecomorphs were monophyletic, as predicted by standing genetic variation (Table 1). We selected only the Green and Large Brown ecomorphs for this analysis due to their lack or minimal evidence of hybridization. None of the 4275 trees retrieved the Large Brown ecomorph as monophyletic, while only one tree (Scaffold 1342 between 1,420,000-1,440,000bp) retrieved the Greens as monophyletic (Table 1). This region included five genes, two of which had hits on NCBI: gene g18405 (*T. kauaiensis* annotation) had a query cover of 100%, an e-value of 1e-103, and a 99.32% identity to the PDE6 gene in the spider *Nephila pilipes*. Gene g18407 (*T. kauaiensis* annotation) had a query cover of 100%, an e-value of 0, and a 76.76% identity to the Gyc32E (Guanylate cyclase 32E like protein) gene in *Argiope bruennichi*.

4 | DISCUSSION

In this work, we investigated whether *Tetragnatha* spiny-leg microhabitat specialists, known as ecomorphs, have repeatedly evolved, and determined the evolutionary processes likely underlying repeated evolution: gene flow, mutation or standing genetic variation (Cerca, 2022; Lee & Coop, 2017, 2019; Stern, 2013). Considering the Green ecomorph as the ancestral state, our findings revealed that the Green ecomorph re-evolved once, the Large Brown and Maroon ecomorphs evolved twice and the Small Brown evolved three times. The evolution of Maroon and Small Brown ecomorphs may have involved ancestral hybridization, while mutation likely drove the evolution of Green and Large Brown ecomorphs, despite a significant degree of incomplete lineage sorting observed in the dataset.

We discuss the evidence for the three distinct processes and conclude by discussing the evolutionary, ecological and genomic factors that may underlie repeated evolution in insular adaptive radiations. Our study contributes to the growing body of evidence demonstrating that repeated phenotypic evolution can be driven by various underlying genomic mechanisms (Alaei Kakhki et al., 2023;

Meleshko et al., 2021; Montejo-Kovacevich et al., 2022; Pease et al., 2016).

4.1 | Evidence for repeated ecomorph evolution in the *Tetragnatha* spiny-legs

Our study is congruent with previous evidence regarding the repeated evolution of spiny-leg ecomorphs (Gillespie, 2004). This is evident from the polyphyletic and paraphyletic placement of ecomorphs observed in various phylogenetic reconstructions (Figures 2 and 3, Figure S1). Additionally, the genetic admixture analyses (Figure 5) indicate that different ecomorphs have different ancestry compositions, providing further support for repeated evolution of the ecomorphs. The phylogenetic reconstructions and the observed patterns of genetic structure are largely congruent with each other and with previous studies using both nuclear and mitochondrial DNA (Gillespie, 2004; Kennedy et al., 2022; Pons & Gillespie, 2003). Although there are minor topological incongruences (e.g. *T. kukuiki* is not being recovered as monophyletic in the ASTRAL reconstruction, Figure 3; and a different placement of *T. perreirai* in the mitochondrial analysis, Figure 2b), the overall results are concordant.

We were unable to include *T. macracantha*, a Green ecomorph species found on the Haleakalā volcano in Maui and on the island of Lana'i, nor *T. kukuhaa*, a Small Brown species found on the Big Island. However, previous research by (Cotoras et al., 2018; Gillespie, 2004; Kennedy et al., 2022) shows that these two species cluster with other Green and Small brown ecomorph species, respectively. Therefore, the observed evolutionary patterns likely apply to this species as well.

4.2 | The contribution of hybridization to repeated evolution in spiny-legs

Our analyses revealed excess allele sharing between distantly related species belonging to the same ecomorph, which is likely to be the result of hybridization. Evidence of hybridization comes from the nuclear-mitochondrial conflicts in phylogenetic reconstructions (Figure 2) and from excess allele sharing analyses where some lineages have more than 10% of whole-genome variants with signatures of excess allele sharing (*T. perreirai* and *T. kamakou*; Figure 3). In the past, hybridization was considered evolutionary noise (Wagner Jr., 1970), but it is now recognized as a key facilitator of speciation

(Marques, Meier, et al., 2019; Meier et al., 2017) and adaptation (Cuevas et al., 2021; Sowersby et al., 2021). Hybridization has the potential to expedite the emergence of an ecomorph in a new geographic area by providing the necessary genetic variation, thus acting as a vehicle for the 'blueprint' for that ecomorph (De-Kayne et al., 2022; Kautt et al., 2020; Richards & Martin, 2017; Sowersby et al., 2021). For instance, if an individual from a given ecomorph disperses to a novel area by chance and hybridizes with one of the lineages present, the introgression of ecomorph-specific genetic variation can lead to the emergence of that ecomorph in the new region, provided an empty ecological niche.

We found evidence of excess allele sharing in comparisons involving species belonging to the Maroon ecomorph. The placement of *T. perreirai* in both nuclear and mitochondrial trees is not topologically congruent, which is a common characteristic of hybridization, although it is not exclusively indicative of it. However, the two maroon species do not currently overlap geographically (*T. perreirai* occurs on the island of O'ahu, *T. kamakou* on Maui and Moloka'i; Figure 1e), and two scenarios could have led to the lack of monophyly of this group in the face of hybridization. First, introgression may have occurred between individuals of *T. perreirai* which moved from O'ahu into Maui Nui and mixed with an ancestral local lineage, resulting in the admixed species that is now *T. kamakou*. This event may have introduced alleles associated with the Maroon ecomorph and opened up the Maroon-niche to the admixed lineage. Alternatively, the Maroon phenotype may have first evolved in *T. kamakou* and was introgressed to O'ahu, leading to the evolution of *T. perreirai*. This scenario implies a back colonization of O'ahu, which is older than Maui Nui. Further data including more genomes, higher coverage, and demographic simulations are necessary to confidently distinguish between these scenarios.

TABLE 1 Analyses of incomplete lineage sorting versus mutation. We obtained 4275 regions of 20,000 bp of the genome with >100 polymorphisms and explored whether the Green and Large ecomorphs were retrieved as monophyletic (expected under a ILS scenario) or polyphyletic (expected under a mutational scenario).

	Green ecomorph	Large Brown
Number of trees where the ecomorph is retrieved as monophyletic	1	0
Total number of trees surveyed	4275	4275

FIGURE 6 D_{XY} estimates for different genomic regions, focusing on (a) Maroon phenotypes, (b) Small Brown (*T. restricta* vs. *T. mohihi*), (c) Small Brown (*T. anuenue/kikokiko/kukuiki/obscura* vs. *T. mohihi*) and (d) Small Brown (*T. anuenue/kikokiko/kukuiki/obscura* vs. *T. restricta*). From left to right: the green-orange compares whole-genome D_{XY} estimates (green) with the D_{XY} for the top 1% positive F_{dM} (orange) in the species pair of interest (same ecotype); the pink-green compares one of the species of interest with another ecotype (Green ecomorph, *T. kauaiensis*), for the same genomic regions where we found 1% positive F_{dM} in the same-ecotype comparison; the yellow-brown compares one of the species of interest with another ecotype (Green ecomorph, *T. kauaiensis*), for the same genomic regions where we found 1% positive F_{dM} in the same-ecotype comparison. Notice that for plotting displays we only included '*T. anuenue*' but this refers to the clade comprising *T. anuenue*, *T. T. kikokiko*, *T. kukuiki* and *T. obscura*. Statistical significance tests were conducted using Wilcoxon tests, with the genomic-level of variation being adjusted to a similar number of samples to the top 1%.
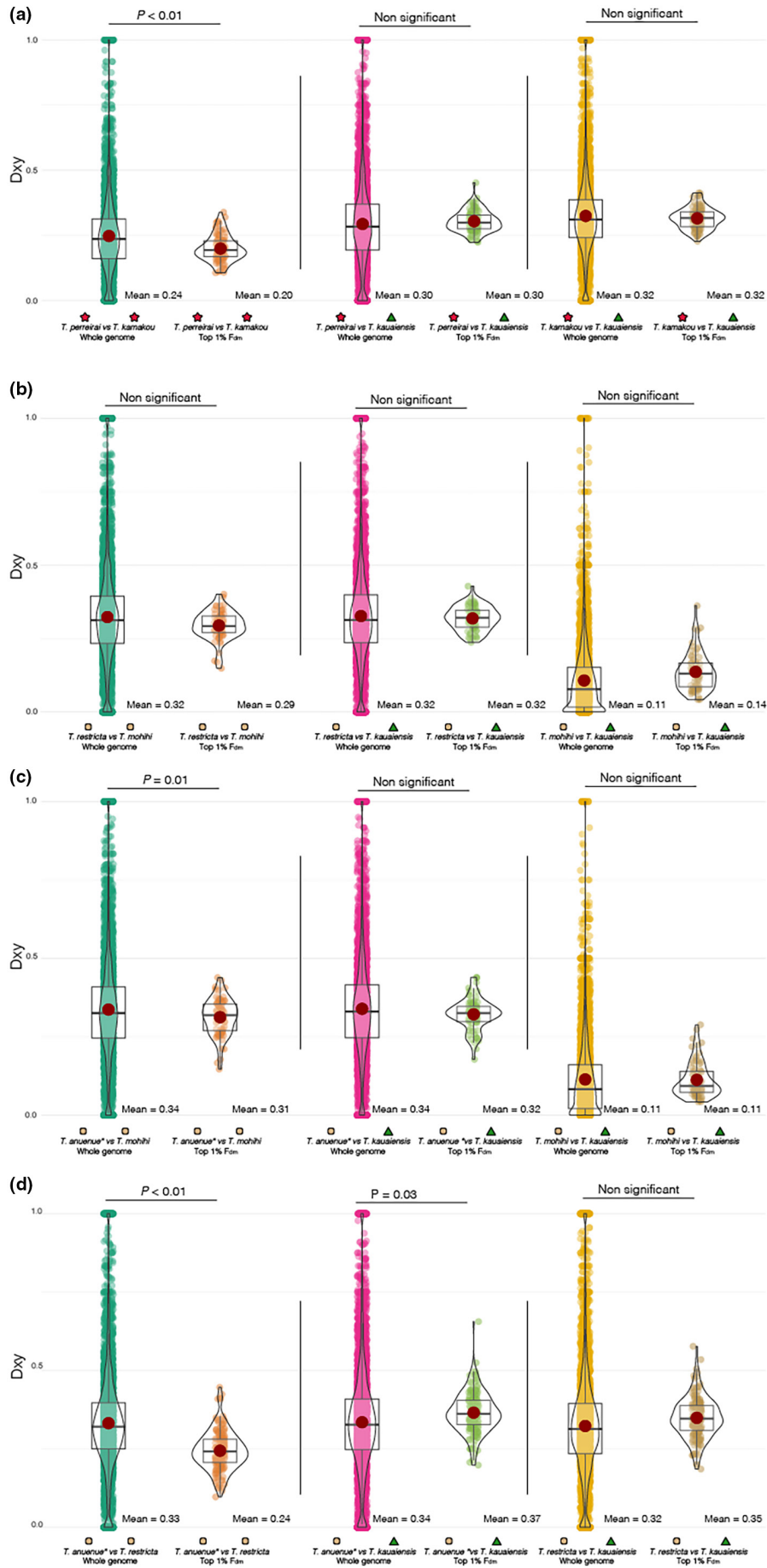
We also found evidence of excess allele sharing between species of the Small Brown ecomorph. Specifically, there is excess allele sharing between *T. restricta*, *T. mohihi* and the clade comprising four Small Brown ecomorph species (*T. anuenue*, *T. kukuiki*, *T. obscura*, *T. kikokiko*). While this signal is particularly clear from the Patterson's D analysis (Figure S2), it is less apparent from the F4 ratio test (Figure 4). This inconsistency is likely due to the different topologies underlying the two tests. In any case, our results contribute to the growing body of research demonstrating that hybridization can be a powerful force in evolution, likely driving repeated phenotypic evolution in the context of an adaptive radiation by the transfer of alleles from one lineage to another (Marques, Lucek, et al., 2019; Meier et al., 2017; Sowersby et al., 2021).

We find that hybridization in the spiny-leg radiation has likely occurred in the distant past (*hereafter* ancestral hybridization). Three factors suggest this. First, admixture analyses indicate that there are very few shared ancestry tracks between different species, even at high levels of K, such as $K=15$, and this suggests that signatures of hybridization may have been diluted over time. Second, while the D_{XY} analyses reveal that the regions with top F_{dM} have lower genomic divergence than the rest of the genome, the homogenizing effect of gene flow would typically lead to considerably lower genomic divergence in regions with high allelic imbalance. Instead, we observed only slightly lower D_{XY} values for these regions, which suggests that hybridization occurred in the past and genomic divergence has accumulated since then. Finally, ancestral hybridization may be likely when considering the present-day distributions of taxa: the maroon ecomorphs live on different islands (Figure 1e), and only *T. restricta* and *T. kikokiko* overlap in the small brown comparison. Therefore, hybridization may have happened between ancestral lineages which predate the current species and actually co-occurred in the same area.

4.3 | The contribution of standing genetic variation to repeated evolution in spiny-legs

Hybridization alone is not sufficient to explain the repeated evolution of every ecomorph. In particular, there is no evidence for excess allele sharing between the two distinct Green ecomorph clades (*T. kauaiensis*, and the clade comprising the lineages *T. brevignatha*, *T. waikamoi*, *T. tantalus*, *T. polychromata*), and only a weak signal of excess allele sharing between the two Large Brown clades (*T. pilosa* and *T. quasimodo*). It is plausible that the repeated evolution of these ecomorphs occurred through mutation or the recruitment of ancestral genetic variation (Barrett & Schluter, 2008; Lee & Coop, 2017, 2019; Stern, 2013). Distinguishing between these two scenarios typically benefits from a focus on selective sweeps and coalescence of variants, which would need higher sequencing coverage and broader population sampling than what was obtained in the current study (Barrett & Schluter, 2008; Lee & Coop, 2017). Although we were unable to obtain such results with low-coverage data, several pieces of evidence allowed us to indirectly infer whether standing genetic variation or mutation have played a role in the repeated evolution of phenotypes.

Standing genetic variation refers to variants that are already present in the genetic pool of a lineage, making them readily available for use (Barrett & Schluter, 2008). If standing genetic variation is responsible for the repeated evolution of ecomorphs, then we would expect to see some regions of the genome under linkage to be monophyletic because these regions would have a single origin but have been selected multiple times. We tested 4275 regions of the genome, corresponding to 8,500,000 bp (8,5 megabases) or approximately 7% of the genome, and only found a single tree where the Green ecomorphs are monophyletic. No trees grouped the Large Brown species as a monophyletic lineage. Despite these results, we found low values of gene and site concordance factors, together with short branches in the phylogenetic reconstructions, which may indicate high rates of incomplete lineage sorting in the radiation, an expected scenario in insular lineages where radiations often arise rapidly (Cerca et al., 2023; Choi et al., 2021).

One potential issue with this approach is that important regions of the genome may have been missed since we focused on only 7% of the genome, and genes associated with each ecomorph's traits could be located in various parts of the genome. Given the variation in environmental tolerance (Hiller et al., 2019), diet (Kennedy et al., 2019), size and colour pigmentation (Gillespie, 1991) among the ecomorphs, it is unlikely that a single gene is responsible for the observed phenotypic changes (Rockman, 2012). For example, the genomic region specific to the Green ecomorph revealed genes involved in light energy conversion (eye phototransduction) and a neurobehavioral signalling factor. Although it may be tempting to attribute the Green phenotype blueprint to these genes, they are likely just one part of a more intricate genetic basis.

4.4 | The contribution of de novo mutation to repeated evolution in spiny-legs

Given the lack of ecomorph monophyly in windows of 5000 bp, and lack of excess allele sharing, mutation appears as an appealing alternative hypothesis to explain the evolution of Green and Large Brown ecomorphs. While it is possible that we missed some genomic signal as the 4275 trees covered only 7% of the genome, the overall lack of monophyly suggests this may not be the case (only one tree out of 4275 was monophyletic for the Green ecomorph). Further research with additional genomes and data will be necessary to fully understand the underlying mechanisms of this evolution.

4.5 | Eco-evo-genomic drivers of ecomorph evolution

A major goal of adaptive radiation research is to disentangle the ecological drivers of species formation (speciation) and adaptation to the environment (adaptation). Spiny-leg *Tetragnatha* spiders are largely confined to mid-elevation wet and mesic forests on Hawaiian volcanoes (1200–1800 m) and diversification has occurred largely within this environment

(Hiller et al., 2019). This suggests that, in addition to the physical barrier between islands imposed by the ocean, the lowland area may act as a strong isolating barrier, as has been shown for many taxa in more stable environments (Janzen, 1967). The spiders' forest habitats have mosaic distributions, and it is likely that the separation between them has triggered a dynamic interplay between natural selection in response to micro-habitat availability and allopatric divergence (Cotoras et al., 2018; Roderick et al., 2012; Vandergast et al., 2004). In particular, ecomorph divergence is likely due to allopatric establishment on different islands and volcanoes, followed by secondary contact with competition between ecologically similar species and hence the accumulation of divergence due to niche differentiation (Cotoras et al., 2018; Schluter, 2000). Then, dispersal and secondary contact of diverged populations may have led to a macroevolutionary character displacement between species, and the evolution of particular eco-morphologies. Our current results add a new element to this model, where hybridization could play a role in facilitating the transfer of genes required to the evolution of some eco-morphologies. However, this is not an exclusive mechanism to acquire the genetic machinery for the expression of those adaptive phenotypes as genetic standing variation and *de novo* mutations may also act as likely sources of genetic material.

5 | CONCLUSION

The whole-genome sequencing of *Tetragnatha* spiny-leg ecomorphs showed that the repeated evolution of ecomorphs in closely related species can result from different evolutionary processes, as some ecomorphs likely arose in the face of gene flow (Small Brown, Maroon), while others likely arose by shared standing genetic variation or mutation (Green, Large Brown).

AUTHOR CONTRIBUTIONS
JC, DDC, THS and RGG obtained funding. JC and RGG designed the study. RGG, DDC, SK, HK and AJW provided samples. JC and LH generated the data. JC analysed the data with contributions on scripting and interpreting the data from CGS, VCB, JM, DDC, CFP, DD and JML. JC drafted a first manuscript, and all the authors contributed to the writing and clarity of the manuscript.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data are available through ENA study PRJEB64196 found at https://www.ebi.ac.uk/ena/browser/view/PRJEB64196. The Code can be found on José Cerca's personal github. https://github.com/jcerca/Papers/tree/main/Tetragnatha_MolEcol.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.