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Research

Evolution

High sexual display trait diversity without measured genetic divergence in a montane hybrid zone involving young species (*Habronattus americanus* subgroup, Araneae: Salticidae)

Tierney Bougie[1,2,3], **Alan Brelsford**[2], **Marshal Hedin**[1,*]

[1]Department of Biology, San Diego State University, San Diego, CA 92182, USA, [2]Evolution, Ecology, and Organismal Biology Department, University of California–Riverside, Riverside, CA 92521, USA, [3]Advanced Genomics Group, National Resilience, San Diego, CA 92121, USA [*]Corresponding author, mail: mhedin@sdsu.edu

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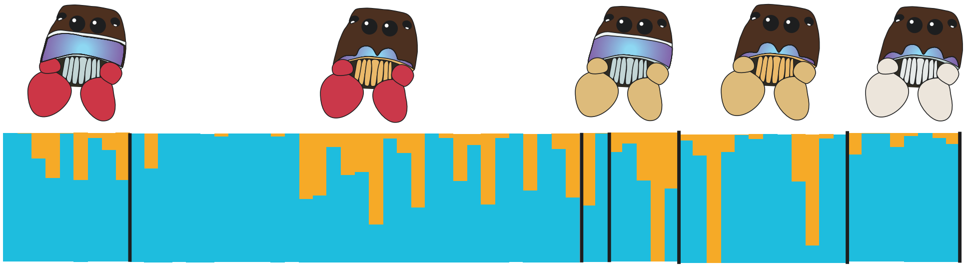
Genetic introgression, allele exchange across species boundaries, is a commonly recognized feature of animal evolution. Under such a paradigm, contemporary contact zones provide first-hand insight into the geographic, phenotypic, and genetic details of introgression. Also, when mate choice phenotypes are conspicuous and variable in hybrids, contact zones provide potential insight into how sexual selection interacts with species boundary maintenance, particularly when postzygotic reproductive isolation is weak. The *Habronattus americanus* subgroup includes several recently evolved jumping spider species, with an estimated age of about 200,000 yr, and substantial evidence for hybridization and introgression. We explored a contact zone involving *H. americanus* (Keyserling, 1885) and *H. kubai* (Griswold, 1979) on Mount Shasta, CA, in alpine habitats that would have been unavailable (under ice) at the Last Glacial Maximum. We characterized morphological diversity within the contact zone, including the fine-scale geographic distribution of hybrid and parental individuals, and assessed genetic variation using ddRADseq data. Combined results indicate a lack of measured genomic differentiation between specimens with distinct morphologies, including individuals with phenotypes of the parental species. We identified a diverse array of hybrid morphologies, with phenotypic evidence for backcrossing, essentially forming a phenotypic bridge between parental taxa. The study area is characterized by more hybrid than parental individuals, with a significantly larger number of red-palped morphologies than white- and/or yellow-palped morphologies; the novel, white-palped phenotype is perhaps transgressive. Overall, these results contribute to a better understanding of the expected ebb and flow of lineage interactions during the early stages of speciation.

Key words: Cascade Mountains, introgression, hybrid zone, phenotypic bridge, sexual selection

Graphical Abstract



Introduction

Many animal biologists, raised on the biological species concept, have long focused on the idea of isolation, both in the speciation process and in defining species. Research over the past 2 decades has now taught us that instead of (or in addition to) isolation, gene flow is an equally important force during and after the animal speciation process (Schwenk et al. 2008, Payseur and Rieseberg 2016, Edelman and Mallet 2021, Dagilis et al. 2022). Plant biologists are long familiar with the concept of syngameons, defined as networks of multiple species connected by gene exchange or multispecies reproductive communities (reviewed in Buck and Flores-Rentería 2022). Phylogenomic evidence for individual animal clades (e.g., genera) now shows that while not always involving *multispecies* networks, interspecific gene flow is a common and recurrent feature of animal evolution, at both ancient and shallow phylogenetic levels. Examples of taxa include whiptail lizards (Barley et al. 2019), *Xiphophorus* (Cui et al. 2013) and *Catastomus* fishes (Bangs et al. 2018), and *Anopheles* (Fontaine et al. 2015), *Drosophila* (Suvorov et al. 2022), and *Heliconius* (Edelman et al. 2019, Kozak et al. 2021) insects.

A shift in perspective regarding the role of gene flow in animal speciation also requires a shift in views of animal hybrid zones. Rather than seeing these zones always as locations where isolated species come into secondary contact and test well-established species boundaries, these zones should be seen as a natural part of a more fluid and perhaps frequent or cyclical process, often involving weakly isolated taxa, or linking a community of evolving species. For example, the ephemeral speciation model (Rosenblum et al. 2012) posits that speciation is rapid and frequent, but that most new species do not persist because of extinction, which sometimes involves lineage fusion at contact. Also, while historical studies of introgression relied mostly upon hybrid zones, because early molecular phylogenetic evidence generally lacked the resolving power to clearly show introgression, the reverse is now often true. Many clades have extensive phylogenomic evidence for historical gene flow, with less direct evidence for modern gene flow in hybrid zones. Having complementary broadscale phylogenomic and contemporary contact zone evidence increases our understanding of the specifics of the gene flow process.

Genomics is obviously playing a fundamental role in understanding gene flow processes at contact, but morphology can also be very important, particularly when interacting taxa have variable and conspicuous mating display traits that could play a role in assortative mating. These situations also inform the interplay between sexual selection, gene flow, and speciation. A common perspective is that sexual selection underlies the rapid evolution of premating isolating phenotypes, which enhances reproductive isolation (reviewed in Servedio and Boughman 2017, Mendelson and Safran 2021). But both empirical studies and theory do not strictly support this notion. In Australian fairy wrens, asymmetrical mating preferences for a colorful secondary sexual feature drive gene and trait introgression in a hybrid zone, diminishing phenotypic differences across subspecies boundaries (Baldassarre et al. 2014). Here, sexual selection is eroding, rather than enhancing, reproductive barriers. Also, when mate choice is frequency dependent and relative rather than absolute, comparatively rare species can be more susceptible to hybridization (Wirtz 1999, Chan and Levin 2005). More generally, theory predicts that sexual selection can act to increase the probability of interspecific gene flow in certain circumstances, certainly when acting as the primary evolutionary force (Servedio and Boughman 2017, Aubier et al. 2019, Irwin 2020). Again, modern contact zones involving taxa which differ in sexual display phenotypes allow for direct investigations of these processes.

The jumping spider genus *Habronattus* F. O. Pickard-Cambridge, 1901, is a compelling lineage to study historical introgression and the nature of hybrid zones in a taxon with remarkable sexual display phenotypes. *Habronattus* is a species-rich genus (>100 described species) that diverged relatively recently—possibly less than 5 million yr ago (Bodner and Maddison 2012). Adult male *Habronattus* have colorful morphological ornamentation and elaborate multimodal courtship behaviors, with many studies demonstrating or discussing sexual selection via female choice acting on these phenotypes (Masta and Maddison 2002, Elias et al. 2005, 2006, Hebets and Maddison 2005, Taylor and McGraw 2013). Even with species differences concentrated in complex mating phenotypes, many *Habronattus* taxa show both morphological and genetic evidence for hybridization and introgression (Maddison and McMahon 2000, Masta 2000, Maddison and Hedin 2003, Hedin and Lowder 2009, Blackburn and Maddison 2014, Leduc-Robert and Maddison 2018, Hedin et al. 2020). The *H. americanus* group is one such clade with documented evidence for hybridization and introgression (Blackburn and Maddison 2014, Leduc-Robert and Maddison 2018, Hedin et al. 2020).

The *H. americanus* group includes a subclade of taxa, the *americanus* subgroup, hypothesized by Hedin et al. (2020) to be about 200,000 yr old based on comparative transcriptomic data. Formally described species in this subclade include *H. americanus*, *H. bulbipes* (Chamberlin and Ivie, 1941), *H. kubai*, and *H. sansoni* (Emerton, 1915), all distributed in mostly montane habitats of western North America. The relatively more geographically isolated *H. waughi* (Emerton, 1926) is found in eastern Canada. Here, our treatment of species in this subclade follows existing taxonomy, as based on patterns of morphological variation (Griswold 1979, 1987). At Mount Shasta, the interacting parental forms are morphologically very distinct, corresponding to the described species *H. americanus* and *H. kubai*. However, more broadly in the subgroup, traditional "species" are not without uncertainties because available phylogenetic evidence does not recover morphologically defined species, and morphology itself is variable to different degrees within described species. Below we highlight these uncertainties, while also specifically emphasizing evidence for the biological reality of the focal species, *H. americanus* and *H. kubai*.

Previous studies have documented hybridization and introgression within the *americanus* subgroup, both between geographical variants of the same recognized species (e.g., different geographic forms of *H. americanus*, Blackburn and Maddison 2014) and between 2 or more different taxonomically described species (Leduc-Robert and Maddison 2018). A phylogenomic analysis focused specifically on this complex recovered clades defined by geographic region, with each regional clade including multiple described species (Bougie et al. 2021). An alternative phylogenomic structure with recognized species as clades (i.e., traditional taxonomy) was explicitly rejected by these data (Bougie et al. 2021). This signal of regional introgression, where groups of adjacent taxa are perhaps acting as connected reproductive communities through time (e.g., see Zhang et al. 2019), appeared to overwhelm a much smaller signal of genomic divergence corresponding to species divergence. In this regard, we see many parallels between *Habronattus* and colorful bird radiations. Recent bird studies have shown extensive background introgression such that recognized morphological species are indistinguishable using traditional molecular markers, with very small regions of genomic divergence relating to species-defining plumage differences, and sometimes adaptive introgression of these plumage genes and traits (e.g., Toews et al. 2016, Wang et al. 2020, Aguillon et al. 2021, Baiz et al. 2021). Given the combined genetic, morphological, and biogeographic evidence, Bougie et al. (2021) hypothesized that Pleistocene climatic fluctuations enabled diverging populations of the *americanus* subgroup to repeatedly come into contact and exchange genetic material, effectively homogenizing genomes, with selection maintaining unique male traits as key species traits (Bougie et al. 2021). Similar secondary contact and hybridization dynamics are found in other montane taxa from the western United States, e.g., in *Lycaeides* butterflies (Nice et al. 2005, Gompert et al. 2010).

Described *americanus* subgroup species currently have mostly nonoverlapping geographic distributions, and almost always, a single geographic location includes one geographic variant or species (see also Blackburn and Maddison 2014). This observation of a single phenotype per location, plus evidence from common garden-rearing experiments (Blackburn and Maddison 2014), suggests that distinctive morphologies in this clade have a genetic basis and are not genetic polymorphisms or environmental polyphenisms. We are not aware of local species sympatry within the subgroup that does not also include evidence for hybridization. Several contact zones between 2 or more *americanus* subgroup species have been identified through our own collecting efforts or those of colleagues (W. Maddison, personal communication); all known contact areas occur at higher elevations in mountains or at high latitudes. This study provides the first formal analysis of such a contact area, involving *H. kubai* and *H. americanus*, on the southern flanks of Mount Shasta, CA (Fig. 1). The last Pleistocene glaciation advance about 17,000–11,000 yr ago covered the south flank of Mount Shasta down to around 1920 m (Christiansen et al. 2017), covering the hybrid zone location (at ~2,200 m), with glacial ice and dating the current hybrid zone location as no older than 17,000–11,000 yr. Of course, we cannot know if Mount Shasta zone dynamics are older than this, with the zone shifting in elevation through time.

The parental species that are interacting on Mount Shasta differ considerably in male morphology, in their geographic distributions, and perhaps also in microhabitat preference (although this has not been formalized). *Habronattus kubai* males possess mostly dark, unmarked ventral first legs, have cheliceral and palpal coverings with golden/yellow pigmented scales, and have a narrow band below the principal eyes (the clypeus) with triangular-shaped medial iridescent scale patches but darker lateral regions (Fig. 2; Supplementary Fig. S1). In contrast, *H. americanus* males possess first legs with ventral stripes, have white-scaled chelicerae but red-scaled palpal coverings, and have a higher clypeus that is iridescent from side to side (Fig. 2; Supplementary Fig. S1). For both species, these forward-facing morphological traits are displayed to visually acute females during a multistage courtship dance that also includes acoustic elements (Blackburn and Maddison 2015, Bougie 2022; D.O. Elias, personal communication). Adult females of these 2 species are very similar (if not indistinguishable) in their muted brown appearance.

Habronattus americanus is relatively broadly distributed in mountainous western North America, including the Rockies, Canadian Rockies, Cascades, and Sierra Nevada mountains (Griswold 1987, Blackburn and Maddison 2014, Bougie et al. 2021). Populations of this species have been found in a range of habitats, including on beaches in British Columbia (Blackburn and Maddison 2015), alpine sagebrush in the Sierra Nevada, and saltgrass flats in central Nevada, among others. Conversely, *H. kubai* has a smaller and apparently fragmented geographic distribution with small pockets of known populations in the Cascade and eastern Sierra Nevada mountains (Griswold 1987; M.H., personal observations). This described species is less geographically and morphologically cohesive than *H. americanus* and could possibly involve multiple taxa, although the Mount Shasta form of *H. kubai* does have a slightly broader distribution both south and north in the Cascade Mountains. Most of our collections of *H. kubai* have come from higher-elevation meadow edge habitats.

Our primary objectives can be summarized as follows: first, we scored several male morphological traits to understand the relative frequency of hybrid versus parental male phenotypes in the study area. Second, we mapped the microgeographic distribution of parental and hybrid male morphologies, also in the context of microhabitat types, to understand the fine-scale geography of the hybrid zone. This information could provide insight into the geographic structure of the zone, e.g., a clinal versus mosaic spatial structure (see Harrison and Rand 1989). Finally, we used ddRAD sequencing to compare patterns found in genomic-scale data with the morphological data. Because the parental species are both part of the "southern Oregon + northern California" regional ddRAD clade from Bougie et al. (2021), we expected them a priori to be highly genetically similar. However, this genomic similarity was previously documented using many fewer RAD loci shared across the entire *americanus* subgroup (e.g., 655 loci with 50% missing data). We reasoned that comparisons of only Mount Shasta samples would include many more ddRAD loci, thus potentially allowing us to detect what is expected to be limited genetic divergence between the parental species.

Materials and Methods

Specimen Collection

The Mount Shasta study site is located just west of Panther Meadows in the Shasta-Trinity National Forest, at an elevation of approximately 2300 m, on the south flank of Mount Shasta. We surveyed for spiders within an approximately 270,000 m^2 area (Fig. 1). Habitat included a southward extending open area of mostly treeless high-elevation pumice field, with herbs and low shrubs, and adjacent high-elevation coniferous forest. A small, paved road winds through the study site. *Habronattus americanus* subgroup members prefer open habitats, so we spent most survey time out of the coniferous

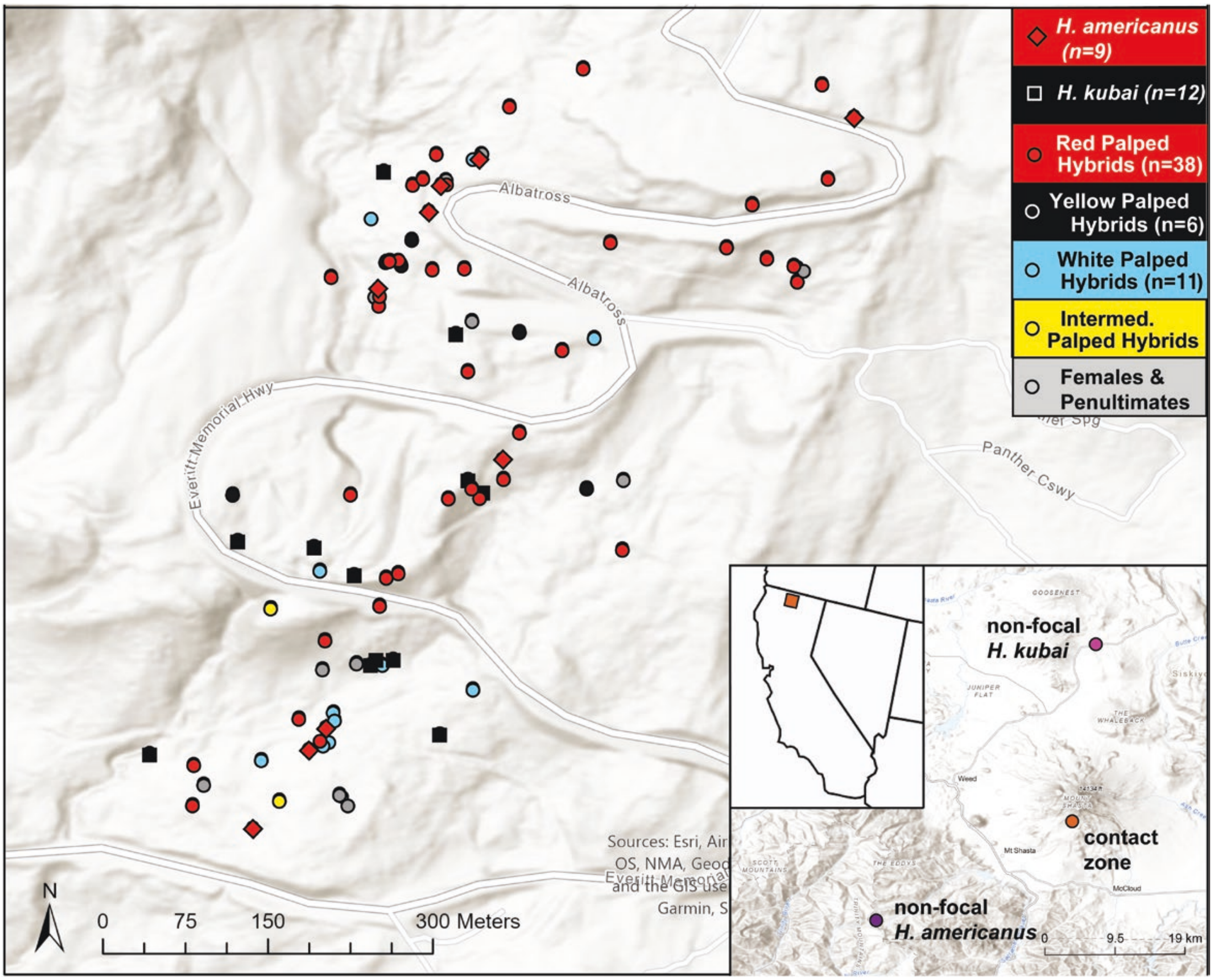


Fig. 1. Map of Mount Shasta contact zone samples. Inset map shows sampling locations for nonfocal *Habronattus americanus* and *Habronattus kubai* populations.

forest. We did not sample the more easterly Panther Meadows proper, with meadow edge microhabitats that we typically associate with *H. kubai*. Special permits were not required to collect spiders from this location.

Evidence of hybridization at this location was previously identified by the collection of male specimens with combined traits of both parental species. In July 2018, a team of 3 persons collected 89 specimens from across the study site, including 78 adult males, 9 females, and 2 penultimate males (Supplementary Table S1). We visually scanned ground habitats at random and attempted to collect all adult males seen (hand-collected into glass vials); because females of the 2 different species cannot be morphologically separated, we limited our collection of adult females. All specimens were georeferenced where collected using a Garmin Etrex 20x handheld GPS device.

Morphological Data Collection and Analysis

Eleven discrete morphological characters were scored for all adult male specimens (Table 1, Supplementary Table S2). Our sample includes individuals described as *H. kubai* and *H. americanus*, with specimens possessing character combinations typical for these species (see description above and Bougie et al. 2021). The remaining specimens are described as *H. americanus* × *H. kubai* hybrids. We scored a subset of morphological characters from Bougie et al. (2021), focusing on characters that varied within the Mount Shasta samples (Table 1). Characters were scored by examining individuals in 100% ethanol under a dissecting microscope. We scored hue (not vibrancy) for some characters, distinguishing red from white from golden/yellow. Acknowledging that color can fade for specimens preserved in ethanol, the specimens that we scored were relatively fresh (2018 collections) and preserved in 100% EToH at −80 °C; we found that most of the pigmentation hue was readily scorable for these specimens.

To summarize morphological variation and identify morphological clusters, we performed a non-metric multidimensional scaling (NMDS) analysis of Bray–Curtis dissimilarity values, using the metaMDS function in the R package Vegan v2.5-6 (Oksanen et al. 2020).

Morphological Characterization of the Contact Zone

To statistically determine whether there are more hybrids than parental morphs, we performed a corrected 1-sided 1-proportion z-test to compare the proportion of hybrids to a target proportion of 0.5, using the function "prop.test()" in R v4.0.3 (R Core Team 2020). The null hypothesis is that the proportion of hybrids is not different from 0.5; the alternative hypothesis is that the proportion of hybrids

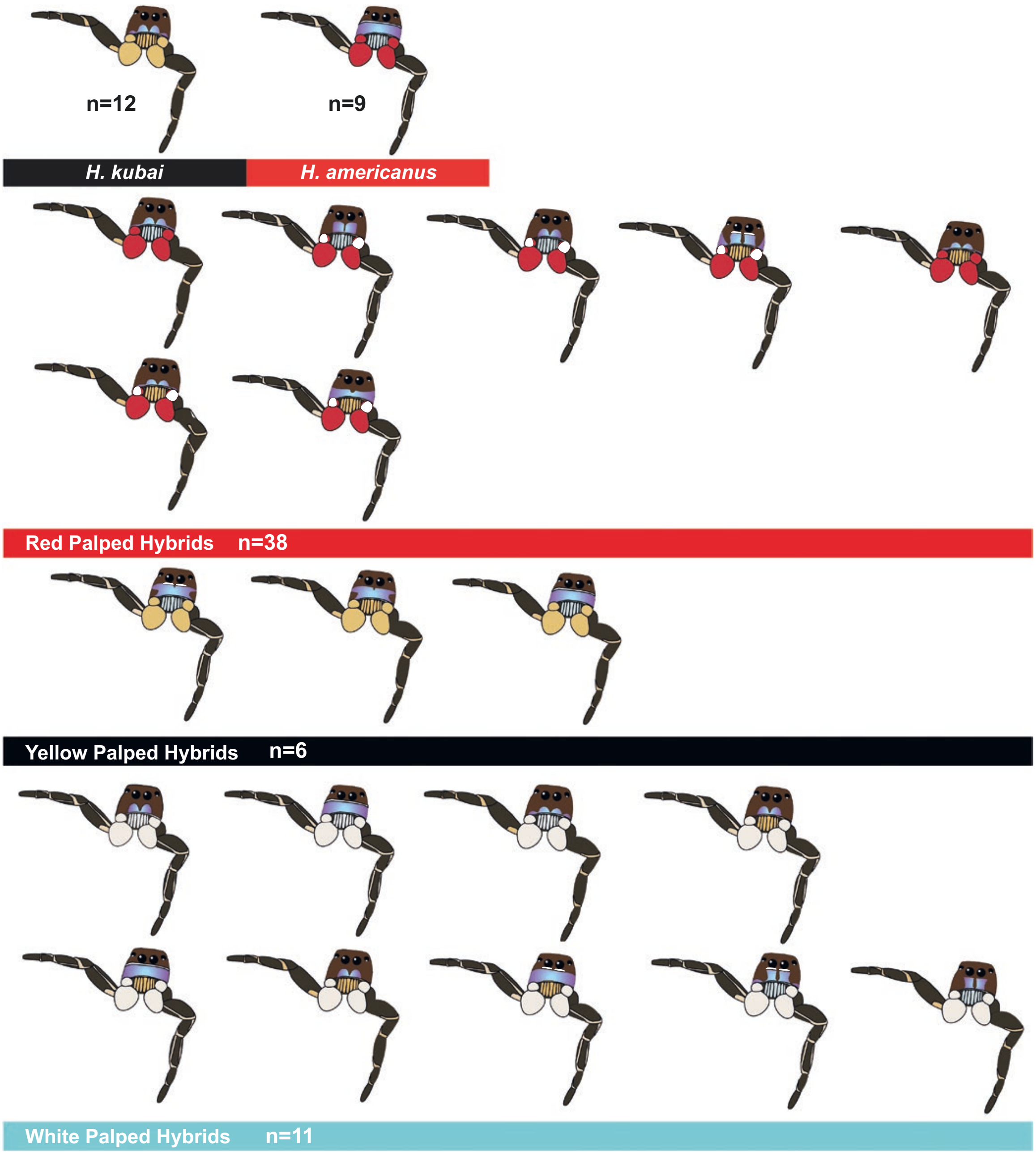


Fig. 2. Cartoon illustrations of all morphological types collected at the Mount Shasta contact zone.

is significantly larger than 0.5, indicating more males with mixed characters than males with parental characters. To test whether the number of differently colored palped individuals was skewed in any direction, we performed 2 χ^2 goodness-of-fit tests. The first test included all male individuals with red, white, or yellow palps, while the second test only included hybrid phenotypes—parental phenotypes were excluded. Both tests shared a null hypothesis that there are equal numbers of red-, yellow-, and white-palped individuals across the study site. To further explore the spatial characteristics of the zone, we mapped the spatial distribution of both parental forms and each hybrid type. For ease of visualization, we binned hybrid morphs into red, white, or yellow palp categories.

Because the sampled contact zone is oriented along an elevational gradient from the south (2270 m) to the north (2,424 m; Fig. 1), we tested for a relationship between palp color and mean elevation. We performed a 1-way analysis of variance (ANOVA) using the function “aov()” in R v4.0.3 (R Core Team 2020), with elevation as the dependent variable and palp color as the categorical independent variable with red, white, and yellow categories. We also performed a post hoc Tukey HSD test to identify whether any comparison of means between specific pairs of palp color groups was statistically significant, using the “TukeyHSD()” function in R v4.0.3 (R Core Team 2020).

To test for a relationship between palp color and habitat type, we recorded the number of red-, yellow-, and white-palped individuals in 2 (barren and shrub) of 4 recorded land cover types (barren, evergreen forest, developed, and shrub; Supplementary Fig. S2), classified according to the National Land Cover Database (NLCD) 2019 Land Cover dataset (Wickham et al. 2021). We only used the barren and shrub type because there are very few samples in the evergreen

Table 1. Scored morphological characters and alternative character states

ID	Description	States
A	Iridescent scales on clypeus—pattern: variable within species, may have iridescent patches, or completely iridescent clypeus	0 = no iridescent scales; 1 = full rectangular; 2 = "m" shaped; 3 = "m" shaped in middle, extends low to sides; 4 = 4 low semicircle broken segments; 5 = 2 irregular iridescent patches separated at the center; 6 = very low iridescent rectangle; 7 = two broken semicircle segments; 8 = 4 connected semicircle segments; 9 = iridescent rectangle halfway up the clypeus and expanding at ends
B	Clypeal covering emarginate: clypeus covered with 2 scale types and/or colors forming a well-marked white transverse band	0 = absent/no white band; 1 = spans entire length of AER; 2 = present only under AMEs
C	Color of noniridescent setae on clypeus, NOT including white transverse band if present	0 = all of clypeus covered in iridescence; 1 = brown; 2 = black
D	Clypeal covering divided in center	0 = absent/not divided; 1 = divided
E	Color of hair pencils/hairs covering chelicerae	0 = blue; 1 = pale/white; 2 = yellow/gold
F	Leg I femur: Color of ventral side	0 = brown/dark; 1 = white/pale; 2 = speckled tan and black; 3 = white stripe/patch, rest brown
G	Leg I femur: pattern	0 = longitudinally striped; 1 = banded at the patella joint, no stripe
H	Leg I tibia: Color of ventral side	0 = brown/dark; 1 = pale; 2 = speckled pale and black; 3 = white patch, rest brown/dark
I	Leg I tibia: pattern	0 = longitudinally striped; 1 = speckled; 2 = plain; 3 = banded at joints, no stripe; 4 = cross between speckled and striped
J	Palpal patella color	0 = uniform, yellow/gold; 1 = uniform, white/pale; 2 = uniform, red; 3 = nonuniform, mostly white/pale, some red
K	Color of hairs covering dorsal cymbium	0 = uniform, yellow/gold; 1 = uniform, white/pale; 2 = uniform, red; 3 = nonuniform, pale white with some pale red hairs

forest and developed types. Additionally, while the land cover shows individuals collected on a developed road, these individuals were not actually collected on the pavement, but nearby. Individuals collected in either evergreen forest or "developed" were recoded to barren or shrub depending on which of the 2 land types was closest to them. Since the observations are binary (barren or shrub), we performed a χ^2 goodness-of-fit test with our expected counts of red = 23.5, yellow = 9, and white = 5.5. The null hypothesis was that there is no relationship between palp color and habitat type.

To test for a correlation between morphology and geographical location, we performed a Mantel test using the Bray–Curtis dissimilarity matrix and a Haversine geographic distance matrix, using the R v 4.0.3 function "mantel()" with 999 permutations in the Vegan v 2.5-7 package (Oksanen et al. 2020). Haversine distances were calculated using the R v 4.0.3 function "distm()" in the geosphere package, version 1.5.10 (Hijmans 2019).

Molecular Data Collection and Analysis

The molecular sample included 72 adult male samples, 2 penultimate males, and 8 females collected at the focal site. We also included a handful of individuals collected from nearby *H. americanus* (Gumboot Lake, ~30 km distant) and *H. kubai* (Grass Lake, ~31 km distant) populations (Fig. 1; Supplementary Table S1). These nonfocal *H. kubai* and *H. americanus* populations are distant enough that it is unlikely they have contributed to the current dynamic near Panther Meadows. At both nonfocal locations, we only found a single species, without evidence for hybrids, but acknowledge that our sample sizes here (less than 20 total individuals per site) may not be large enough to detect rare hybrids.

Two to 3 legs were used for DNA extraction and performed using the Qiagen DNeasy Blood & Tissue protocol (Qiagen, Valencia, CA). DNA quality and quantity were evaluated using gel electrophoresis and a Qubit Fluorometer, respectively. We used double-digest restriction-site-associated DNA sequencing (ddRADseq) to gather genomic-scale data. We used the protocol described in Brelsford et al. (2017), using SbfI and MseI enzymes—a combination that increases sequencing depth while accounting for large *Habronattus* genome sizes (~5.6 Gb, Gregory and Shorthouse 2003). Sequencing was completed at Novogene using 150PE reads on an Illumina Hiseq 4000 platform.

Raw sequence read data were demultiplexed using Stacks v2.5.0 under default settings. After demultiplexing, the reads were processed using Stacks with the --bound_high flag set to 0.05, --min-maf flag set to 0.05, and --min-samples-overall to 90 to require 90% of individuals across the dataset to process a locus. All other parameters were left as default.

We conducted a Structure 2.3.4 (Pritchard et al. 2000) analysis under the admixture model using 2,182 unlinked SNPs, including both focal and nonfocal site samples. Structure was run from K 1 to 4, each replicated 3 times using the package Rrunstruct (Anderson 2015) and function "structure_runs()" in R v4.0.3. To identify possible genetic divergence between different morph types, we estimated pairwise F_{ST} between palp color types using all ddRAD loci (5,873 SNPs), conducted in Arlequin v3.5.2.2 (Excoffier and Lischer 2010). We calculated F_{ST} using different palp-colored individuals as "populations" and a separate analysis using the 3 different palp-color hybrid groups and the 2 parental morphs as "populations." Females, 2 intermediate-palped males, and individuals from nonfocal sites were excluded from these analyses.

We tested for a correlation between Haversine geographical distances and Euclidean genetic distances using a Mantel test, with the R v 4.0.3 function "mantel()" with 999 permutations in the Vegan v 2.5-7 package (Oksanen et al. 2020). Euclidean genetic distances were calculated on a matrix of 6,359 unlinked SNPs using the "dist()" function in the R package stats (R Core Team 2020). To

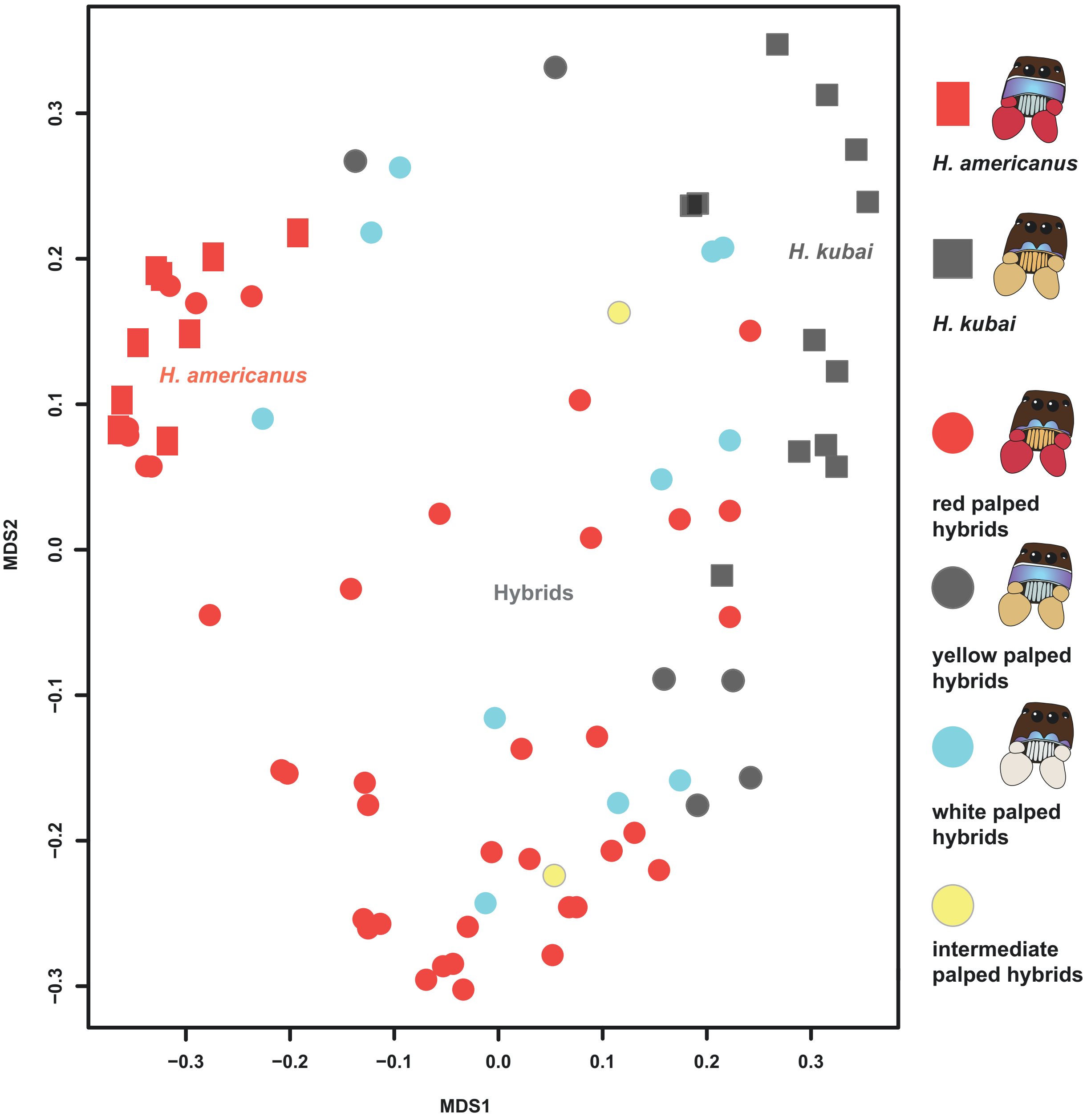


Fig. 3. Results of non-metric multidimensional scaling (NMDS) analysis of morphological data matrix.

test for a correlation between genetic and morphological distance, we performed an additional Mantel test using the same R function as above between the Bray–Curtis dissimilarity matrix of morphological characters and Euclidean genetic distances.

Results

Morphological Data and Analyses

The scored morphological character matrix for all specimens can be found in Supplementary Table S2; all voucher specimens have been deposited in the SDSU Terrestrial Arthropods Collection (SDSU_TAC). We identified 19 different character combinations of hybrid morphologies, defined by different combinations of palp color, cheliceral hair bundle color, leg I pattern, and clypeus iridescence pattern (Fig. 2; Supplementary Fig. S1). The NMDS plot supports a clear morphological separation of parental species and includes a group composed of hybrids that encompasses a large area of the plot falling between the *H. kubai* and *H. americanus* clusters (Fig. 3). The hybrid cluster is variable, demonstrated by the wide area of the group on the plot. While we qualitatively binned hybrids by palp color, there is no evidence for separate hybrid palp-color NMDS clusters. There is some overlap between the hybrid and parental groups, suggesting that some hybrids possess trait combinations that are more like either parental type. For example, an individual possessing mostly *H. americanus* traits, but with one trait belonging to the *H. kubai* parental type might be placed closer to the standard *H. americanus* cluster.

The proportion z-test revealed that there are more phenotypically defined hybrid individuals than parental forms in the contact zone, Z_1, $P = 7.402\ e^{-0.5}$ (Supplementary Table S3). A χ^2 goodness-of-fit test showed that the number of individuals of each palp color are not at equal frequencies (Supplementary Table S4;

$\chi^2_2 = 28.7632$, $P < 0.00001$), with a significantly larger number of red-palped individuals than white- and/or yellow-palped individuals. When conducting the χ^2 goodness-of-fit test only using hybrid specimens (no parental morphs), we also found that individuals of different palp colors are present at significantly different frequencies ($\chi^2_2 = 20.468$, $P < 0.00001$; Supplementary Table S5). These results should be considered with some caution as an assumption of the χ^2 goodness-of-fit test is having a random sample; we believe our sampling was random across the hybrid zone, but it is possible that spiders with brightly colored red palps are easier to spot (or catch) for collectors.

The ANOVA of elevation and palp color identified a significant difference between the mean elevations of each palp color ($F_2 = 5.143$, $P = 0.0081$; Fig. 4). The post hoc Tukey HSD test recovered a significant relationship only between the white- and red-palped individuals ($P = 0.0149$; Fig. 4). Other pairwise comparisons were not significant, implying that the main driver of the significance of our ANOVA is the elevation differences between red- and white-palped males.

The χ^2 goodness-of-fit test to identify a relationship between palp color and 2 land cover types was significant ($\chi^2_2 = 12.4103$, $P < 0.002$; Supplementary Table S6). These results and the ANOVA elevation results should be viewed cautiously since land cover type changes along the elevational gradient. As such elevation and land cover differences may be confounded. The Mantel test to identify a correlation between multivariate morphology and geographical location was not significant ($r = -0.0496$, $P = 0.916$).

Genomic Data and Analyses

ddRADseq data were recovered for 85 individuals, with raw reads available at the Short Read Archive (BioProject ID: PRJNA1046142). Data matrices and analysis results are available at Dryad (https://doi.org/10.5061/dryad.t76hdr86q).

STRUCTURE results indicate a lack of genetic structure across different morph types within the contact zone and including nonfocal populations (Fig. 5). The optimal K value was estimated as $K = 2$, using the Evanno method (Evanno et al. 2005), but was estimated as $K = 3$ using the Prob($K = k$) method as described in Pritchard et al. (2000). Given the lack of confidence in estimating a best K, we show ancestry coefficients for all values of K tested (1–4).

F_{ST} values confirmed a lack of genetic differentiation between males with differently colored palps. F_{ST} values for comparisons between 3 colored palp types, not including parental specimens, were all below 0.005 (Table 2). F_{ST} values for comparisons between the 3 hybrid palp colors including parentals ranged between 0.0215 and 0.0056 (Supplementary Table S7). A Mantel test to identify a correlation between genetic and morphological distance was not significant ($r = 0.0422$, $P = 0.189$).

Discussion

High Male Sexual Display Trait Diversity

Analyses revealed high male sexual display trait diversity within the Mount Shasta hybrid zone and significantly more hybrid individuals than parental individuals (Supplementary Table S3). We qualitatively

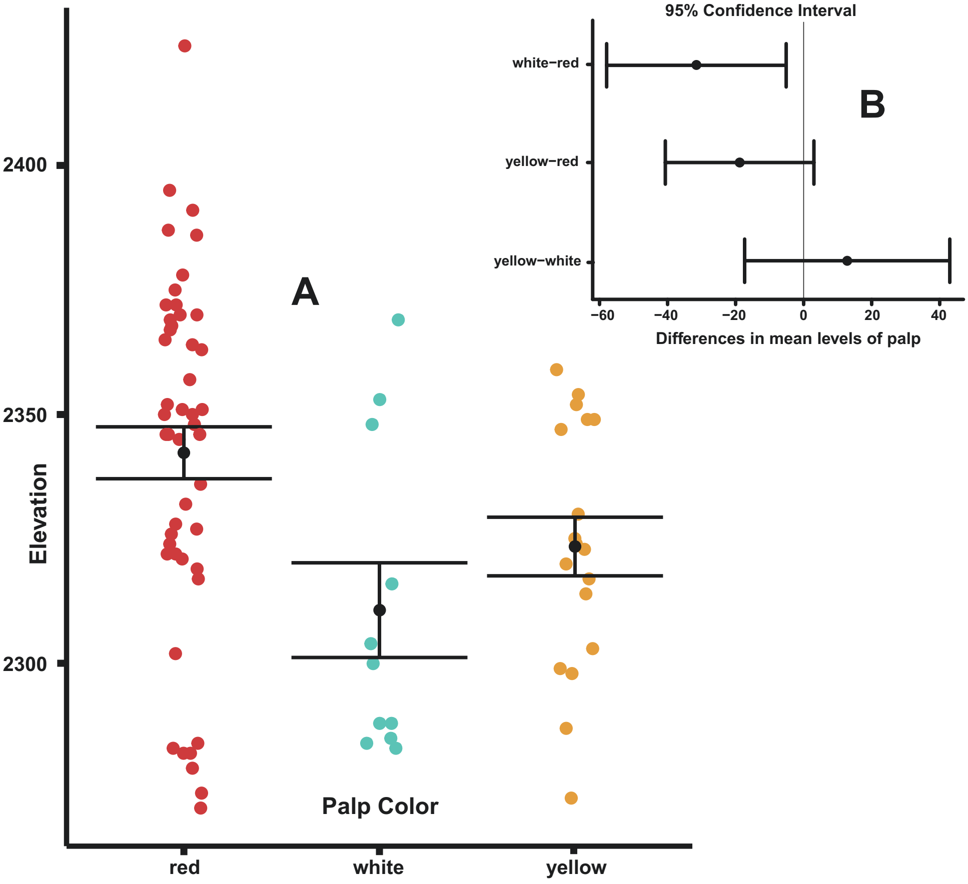


Fig. 4. (A) Scatter plot of sample elevations, grouped by palp color. (B) Graph of Tukey post hoc test.

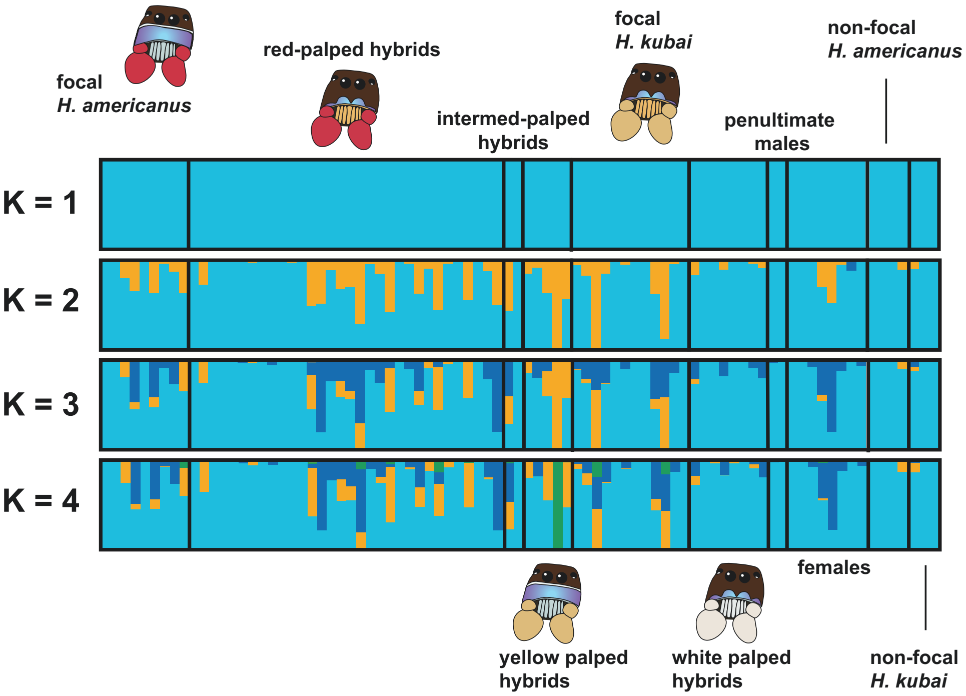


Fig. 5. STRUCTURE admixture proportions for values of $K = 1$–4 organized by phenotypic category. Individuals in each category are organized left to right by increasing elevation.

Table 2. Pairwise F_{ST} estimated using all ddRADseq loci. *P*-values in parentheses.

	Red	White	Yellow
Red	0		
White	0.0090 (0.270 ± 0.036)	0	
Yellow	0.0061 (0.459 ± 0.050)	0.0099 (0.568 ± 0.024)	0

placed hybrids into 19 categories, based on different trait combinations (Fig. 2). The high number of distinct combinations suggests that these traits are encoded by genes in multiple independently segregating linkage groups, otherwise there would be groups of certain traits that always appear together, which is not observed. Also, the wide range of trait combinations suggests a mix of admixed (F_1) and backcrossed hybrids, in addition to parental forms.

Several other studies have shown extensive phenotypic variation in contact zones involving young species. The classic northern flicker hybrid zone, with evidence for random mating and high hybrid fitness, includes the full range of possible plumage combinations across 6 colored plumage patches (Moore 1987, Aguillon et al. 2021). Another bird example includes *Setophaga* warblers, where hybrids display a high diversity of colorful plumage patch trait combinations, with limited assortative mating (e.g., Brelsford et al. 2017). In a study of *Xiphophorus birchmanni* and *X. malinche* hybrids at Calnali (Mexico), Rosenthal et al. (2003) found extensive introgression of parental forms, and a large recombinant array of male sexual trait morphologies, without evidence for assortative mating. However, in this same species pair at different locations, the strength of positive assortative mating varies and sometimes prevents hybridization (Schumer et al. 2017), speaking to the context dependence of prezygotic isolation. The Mount Shasta contact area adds another system demonstrating high morphological diversity in a hybrid zone.

Available evidence suggests that postzygotic isolation in *Habronattus* is weak. This includes evidence from matings across divergent populations or species that result in viable offspring (Masta and Maddison 2002, Blackburn 2013; D.O. Elias, personal communication). And although adult male *Habronattus* commonly attempt to mate with heterospecifics (Taylor et al. 2017), standard dogma might predict that prezygotic isolation resulting from positive assortative mating (sexual selection) would limit hybridization, particularly in a group dominated by colorful and complex male sexual displays. Also, prezygotic isolation is generally thought to evolve prior to postzygotic isolation (references in Schumer et al. 2017, Irwin 2020), early in speciation, and thus relevant to the young species studied here. These expectations are not borne out by the Mount Shasta data, and in fact, these expectations are not strictly consistent with theory.

Irwin (2020) used simulations to understand how positive assortative mating influences population blending when 2 differentiated populations come into secondary contact (i.e., a hybrid zone). Irwin modeled “moderately strong” assortative mating, such that a species A female was 10 times more likely to accept a contype than a heterotype (“10 times assortative mating”). Under these parameters, and because of positive assortative mating, occasionally produced F_1s mated with other F_1s, and because F_1s were more attractive than heterotypes in this model, backcrossing also occurred. After 50 simulated generations, basically all combinations of intermediate forms were found, forming what Irwin (2020) termed a “phenotypic bridge” between parental forms. These results contrasted with a postzygotic isolation simulation (10% hybrid fitness reduction, no assortative mating) that resulted in a much narrower zone with fewer hybrids. Irwin also showed that as the number of loci encoding the assortative mating phenotype increased (from 1 to 9), the expected width of the zone also increased. In general terms, the Irwin (2020) simulations show how strong *but incomplete* assortative mating is ineffective in preventing hybridization when hybrids are fit. Intriguingly, several of these theoretical findings match empirical observations at Mount Shasta (and in *Habronattus* more generally), including weak postzygotic isolation, imperfect assortative mating (Hebets and Maddison 2005, Elias et al. 2006, Blackburn 2013), a relatively broad zone, and the formation of a phenotypic bridge. It would also be interesting to understand how the short breeding seasons at high elevations on Mount Shasta might impact mate search costs and female choosiness in this system (Servedio and Hermisson 2020).

Lack of Measured Genetic Divergence

In contrast to morphological patterns, ddRADseq data show very little genetic structure between different morphs (Table 2; Fig. 5). While the Evanno method recovered an optimal $K = 2$ and the Prob($K = k$) method recovered an optimal $K = 3$, these results should be considered carefully. The Evanno method is incapable of recovering an optimal K of 1 (Evanno et al. 2005)—which seems to be the most likely scenario—and a $K = 3$ seems very unlikely considering the degree of genomic homogeneity in hybrid zone individuals themselves and across most *americanus* subgroup species (Bougie et al. 2021). F_{ST} between different palp-colored individuals further supports a lack of genetic structure. Blackburn and Maddison (2014) used AFLPs to measure genomic divergence among geographic forms of *H. americanus*, which differ strongly in male color morphology, and also recovered a $K = 2$, with overall low levels of genomic divergence, contrasting with conspicuous male display differences.

Lack of measured genetic structure across morphs in the Mount Shasta hybrid zone supports the hypothesis that a small number of loci are responsible for male morphologies in the group and our ddRADseq data were simply unable to detect these loci. These findings mirror more broadscale patterns between morphological and genetic diversity in members of the *americanus* subgroup (Bougie et al. 2021), where a phylogenomic analysis using ultraconserved elements and ddRAD data recovered clades (each including multiple described species) defined by geographic region. And although we did recover many more loci than in Bougie et al. (2021), our ddRADseq loci still only covered a miniscule fraction of what is predicted to be a very large genome. For example, given a mean locus length of 182 bp, 2,182 loci, and an estimated *Habronattus* genome size of ~5.6 Gb, the predicted proportion is $\frac{(182 \times 2{,}182)}{5{,}586{,}000{,}000\ \text{bp}} \times 100 = 0.0071\ \%$. This fraction could be larger depending on recombination rates and linkage of RAD loci to other regions of the genome, but these are currently unknowns.

Spatial Structure of Contact

The Mount Shasta contact zone is not obviously clinal, with parental forms on opposite sides, but rather includes a mixture of hybrids and parental forms across the area sampled (Fig. 1). Based on geographic distributions elsewhere in the *americanus* subgroup, we might have predicted that pure *H. kubai* would be found at higher elevations, with pure *H. americanus* at lower elevations, but we found no evidence for such a pattern. The spatial structure also does not conform obviously to a mosaic zone, which is classically defined as an area of secondary contact between species that differ in habitat utilization, with these habitats themselves distributed in a patchwork or mosaic pattern (discussed in Harrison and Rand 1989, Gompert et al 2010). Our prediction would be that pure *H. kubai* would occupy meadow edge habitats, e.g., Panther Meadows just east of the area that we sampled. Such a pattern is not obvious from our results, but our sampling design did not directly address this possibility.

A mixture of parental and hybrid types is also found at Bunny Flats, a lower-elevation location about 4 km southwest of Panther Meadows (M.H., T.B., personal observations), again characterized by an open pumice field with low shrubs and herbs, surrounded by conifer forest. Whether or not spider populations at Bunny Flats are contiguous with Panther Meadows is unknown. We have not sampled elsewhere on the south flanks of Mount Shasta to understand whether and where "pure" populations of either parental species are found, a situation that requires further scrutiny. Also, regarding spatial scale, these spiders are not strictly sedentary, so the spatial structure measured is not expected to capture a more long-term pattern. Blackburn and Maddison (2015) studied a Canadian population of *H. americanus* and found that adult males cover more space than adult females, the former approximately 10–20 m/h, the latter about 2–4 m. Our impression is that the dispersal distance of these spiders is small relative to habitat patch size (e.g., alpine meadows vs. "other"), but this requires formal study.

Although the microgeographic distribution of parentals versus hybrids does not appear to be clinal or associated with larger habitat patches, there is a significant difference in mean elevations of red- versus white-palped males (Fig. 4). Our land type χ^2 goodness-of-fit test revealed a similar pattern in that red-, white-, and yellow-palped individuals are not at identical frequencies within the 2 different land types, and the transition from one type to the other follows an elevational gradient (Supplementary Table S6). White-palped individuals are more common at lower elevations and are mostly concentrated in shrub habitats, while red-palped individuals are concentrated in mid and high elevations and prefer bare microhabitats, although there were still many red-palped males collected at lower elevations (Fig. 4, Supplementary Fig. S2).

A Common Female Preference for Red?

The Mount Shasta contact zone has more red-palped individuals than white- or yellow-palped males (Supplementary Table S4). Additionally, there are more red-palped hybrids that display mostly *H. kubai* traits ($n = 13$) than yellow-palped hybrids that display mostly *H. americanus* traits ($n = 1$), a pattern that may indicate evidence for selection favoring red-colored palps. Red coloration of male courtship traits is observed in many *Habronattus* species groups (Griswold 1987, Maddison and Hedin 2003, Bougie et al. 2021) and likely evolved independently across the larger phylogeny. Zurek et al. (2015) described a red spectral filter within *Habronattus* that enables trichromatic vision via the addition of sensitivity to red hues, and it appears that this mechanism is a general feature of the genus (Zurek et al. 2015). Also, red coloration in male *H. pyrrithrix* improves courtship success when in high-light conditions (Taylor and McGraw 2013), where spectral filters should be functional. Taylor et al. (2011) also found that adult male red facial patches in *H. pyrrithrix* were redder when preceding juvenile stages were fed higher-quality diets (i.e., condition dependent). Both latter studies indicate that red could be a target of sexual selection via female choice in at least some species of *Habronattus*.

It is possible that the abundance of red-palped males in the Mount Shasta hybrid zone reflects a common female preference for red phenotypes in the broader *americanus* subgroup. Behavioral experiments are needed to better understand the importance of the red palps and whether it is a preferred trait to females in the hybrid zone. However, if red phenotypes increase the propensity for localized hybridization and introgression, this might shed light on patterns seen more broadly in the *americanus* subgroup.

Habronattus americanus has a larger geographic distribution than any other species in the subgroup and occupies a greater variety of habitats (Griswold 1987, Blackburn and Maddison 2014, 2015, Bougie et al. 2021, iNATURALIST 2023). Adult males of this species are not monomorphic in appearance across this broader distributional range, and in particular display different amounts of red across geography. This includes male populations with only red pedipalps, populations with both red palps and ventral first legs, and populations with red palps, first legs, chelicerae, etc. (e.g., the P, PL, and PLC forms of Blackburn and Maddison 2014). These adult male ornaments are forwards-facing and, again, displayed to females during a visual and vibratory courtship dance (Blackburn and Maddison 2015, Bougie 2022).

Other taxonomically recognized species in the *americanus* subgroup have relatively more restricted geographic distributions and appear to occupy fewer microhabitats (Griswold 1987, Bougie et al. 2021, iNATURALIST 2023). Some taxa are also curiously disjunct, while the range of *H. americanus* is broader and more continuous. For example, a population of *H. kubai* from far eastern Nevada is separated from Californian Sierran *H. kubai* populations by only intervening *H. americanus* populations. Similarly, *H. sansoni* from northern NM and south-central CO are separated from disjunct northwestern populations by intervening *H. americanus* populations. Most other species within the *americanus* subgroup also lack red courtship ornaments. Exceptions include "red" *H. sansoni* populations (in southern Canada and central Colorado), with other *H. sansoni* populations having white or cryptically colored male courtship traits.

Overall, the distribution of the *americanus* subgroup might be portrayed as including a single geographically variable, lower-elevation, generalist, red-bearing taxon (*H. americanus*), with other species found in smaller habitat pockets, often at higher elevations or latitudes. If throughout the divergence history of the subgroup *H. americanus* populations have come in secondary contact with other subgroup taxa, perhaps hybridization was driven by a shared female preference for the red-based traits of *H. americanus*. We note that almost all known hybrid zones between *americanus* subgroup members involve *H. americanus* and that these known zones are concentrated at high elevations. Also, this "*sea of red*" hypothesis might help explain the disjunct distributions of the taxa highlighted above, which instead of being naturally fragmented, are perhaps disjunct because intermediate populations have been replaced by red *H. americanus*. This replacement scenario implies extinction via hybridization, but complete replacement is not required by the hypothesis. For example, genomic introgression to varying degrees might explain the extensive geographic variation within *H. americanus*, including populations that appear to represent stabilized hybrid populations (e.g., central Colorado populations, Bougie et al. 2021). The phylogenomic results of Bougie et al. (2021) are consistent with the "*sea of red*" hypothesis in that *H. americanus* is the only described species found in *all* regional genomic clades.

If the red palps of *H. americanus* males have indeed increased propensity for hybridization throughout the divergence history of the subgroup, we may eventually be able to use pigmentation loci to trace evolutionary history, as demonstrated in several bird studies (e.g., Wang et al. 2020, Aguillon et al. 2021).

A Transgressive Phenotype?

Mount Shasta hybrids share multiple traits from parental species, but a new trait not found in either parental species was also discovered: pure white pedipalps (Fig. 2, Supplementary Fig. S1). We view these white palps as a transgressive phenotype, defined as a novel hybrid trait that lies outside of the phenotypic range of parental species (Slatkin and Lande 1994, Rieseberg et al. 1999, Stelkens and Seehausen 2009). A white-palped morphology is not known in either parental species, across their respective distributions, and is otherwise rare in the *americanus* subgroup. The most phenotypically similar condition is found in white-palped *H. sansoni*, with the closest known populations found in the Washington Cascade mountains. *Habronattus bulbipes* populations are found near Mount Shasta in the more westerly Klamath-Siskiyou Mountains, but all populations that we are aware of have "dirtier" palps, tending toward yellow.

The appearance of a novel trait indicates another potentially important role for introgression in the evolution of this species complex, in addition to the hypothesized "sea of red" dynamics discussed above. Here, gene flow is transmitting ornamentation alleles between populations, but this gene flow (and later recombination) is also potentially creating novel traits. Brower (2013) describes a similar mechanism to explain high wing pattern diversity in *Heliconius* butterflies but suggests that the initial evolution of wing patterns was driven by Müllerian mimicry instead of sexual selection. It is possible that similar mechanisms drove the appearance of novel phenotypes in other *Habronattus* species complexes, such as the *H. pugillis* complex in the Arizona sky islands (Maddison and McMahon 2000). More generally, new phenotypes resulting from hybridization have been shown to spark speciation and rapid radiation events (e.g., Mavárez et al. 2006, Lamichhaney et al. 2018, Powell et al. 2021).

Conclusions

Bougie et al. (2021) argued, based on phylogenomic evidence, that the *americanus* subgroup may be best described as a complex of closely related taxa, evolving together through time via occasional or cyclical gene exchange (He et al. 2019, Zhang et al. 2019). In this sense, the Mount Shasta contact zone might represent a microcosm of processes that we hypothesize have been recurring during the divergence history of the *americanus* subgroup. The current dynamics at Mount Shasta highlight the evolutionary consequences when young species are brought into contact.

Mount Shasta populations should be more thoroughly studied to further understand diversification dynamics in this system. Behavioral experiments could measure female preference functions within and outside of the hybrid zone and test whether red phenotypes are favored. Comparative whole-genome sequencing has the potential to identify the differentiated areas of the genome that we hypothesize correspond to male display traits (e.g., Toews et al. 2016). Coupling behavioral data with new genomic data could paint a picture of how introgression has shaped evolution in young species experiencing potentially strong sexual selection.

More generally, *Habronattus* has the potential to become a unique model to understand the interplay of sexual selection and rapid species radiation in an invertebrate taxon. The genus is species rich and remarkably phenotypically diverse, with a robust phylogenomic framework, well-developed behavioral tools, and admixture that allows the study of genotype–phenotype relationships. The major constraints at this time are the very large *Habronattus* genomes, but as sequencing technology continues to evolve, this hurdle might be overcome.

Supplementary Material

Supplementary material is available at *Insect Systematics and Diversity* online.

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Author Contributions

Tierney Bougie (Conceptualization [Equal], Data curation [Equal], Formal analysis [Equal], Funding acquisition [Equal], Investigation [Equal], Methodology [Equal], Project administration [Equal], Resources [Equal], Supervision [Equal], Validation [Equal], Visualization [Equal], Writing—original draft [Equal], Writing—review & editing [Equal]), Alan Brelsford (Conceptualization [Equal], Methodology [Equal], Project administration [Equal], Resources [Equal], Software [Equal], Supervision [Equal]), and Marshal Hedin (Conceptualization [Equal], Data curation [Equal], Funding acquisition [Equal], Investigation [Equal], Methodology [Equal], Project administration [Equal], Resources [Equal], Software [Equal], Supervision [Equal], Validation [Equal], Visualization [Equal], Writing—original draft [Equal], Writing—review & editing [Equal])

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