Reconciling genomic and ecological species delimitation using a confusing group of butterflies

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Abstract

Species delimitation is essential to understanding and categorizing our planet's biodiversity, particularly amidst rapid changes to environmental conditions and natural landscapes. However, the process of speciation is heterogenous and often complex, and robust characterization of species boundaries has remained a challenge for many taxa. Recent advances in both genomics and ecological modelling have been a boon for research focused on population dynamics, and present new, multidisciplinary opportunities for clarifying species boundaries in taxa that have been difficult to classify otherwise. Here, we present an approach to combining ecological niche models with next-generation sequence data to aid in integrated species delimitation. We apply this approach to the Speyeria atlantis-hesperis (Lepidoptera: Nymphalidae) species complex, which is notorious for its muddled species delimitations, morphological variation and mito-nuclear discordance. Using genomic SNPs, we recovered substantial divergence, not only between S. hesperis and S. atlantis, but also within S. hesperis, which may be attributed to a combination of past introgression with another species, S. zerene, and post-glacial range expansion. We then applied niche modelling to assess ecological divergence and barriers to gene flow among the recovered genomic lineages. Results of these analyses suggest that adaptation to ecological conditions is hindering contemporary gene flow between northern and southern populations of S. hesperis, contributing to and reinforcing their genetic integrity. We suggest that the current species delimitation of S. hesperis should be revised, and demonstrate the utility of an approach to integrated species delimitation that combines ecological and genomic data and reconciles related species concepts.

Keywords:

Speyeria , ecological speciation, habitat suitability, species delimitation, population genomics, introgression

Introduction

Species delimitation has increasingly greater biological, social, economic, and political consequences amidst the rapid habitat and biodiversity losses of our planet (Coates *et al.* 2018; Stanton*et al.* 2019). While species comprise the operational units of modern conservation initiatives (Mace 2004; Magurran 2013; MacDonald et al. 2017; IPBES 2019), the procedures employed in species delimitation and identification suffer from a number of limitations, in part because the processes of speciation are affected by diverse historical, genetic, ecological, and stochastic factors (Sites & Marshall 2003; Wiens 2004; de Queiroz 2007; Wilkins 2009; Loera et al. 2012). This has led to a plethora of species concepts that differ in their emphases on morphological, genetic, or ecological information for resolving divergences and distinguishing species, each of which may produce inconsistent or contradictory species assessments (Mayr 1942, 1957, 1963; Mayden 1997; Wiens 2004; de Queiroz 1998; Freudenstein *et al.* 2016).

Assessing species boundaries can be particularly challenging for taxa that are not easily morphologically distinguished or have superficially similar habitat preferences. In this context, quantitative comparisons of

genomic and ecological divergences can aid in the resolution of historical and ongoing speciation events, particularly for allopatric or parapatric sister species (Carstens *et al.* 2013). Furthermore, the integration of these two sources of information permits insight into whether ecologically-based divergent selection has contributed to speciation and reinforcement of a species' genomic integrity (Sperling 2003; Graham *et al.* 2004). For example, significant niche divergence between sister species would suggest that ecologically-based divergent selection is a principal process underlying speciation (i.e., ecological speciation), while niche conservatism suggests that other processes are implicated (e.g., speciation without selection or mutation-order speciation, *sensu* Nosil 2012). Incorporation of ecological information into genetic species delimitation frameworks can thereby provide valuable inferences on modes of speciation and strengthen phylogenetic inferences when traditional approaches have not convincingly done so.

The development of high-throughput DNA sequencing techniques such as RADseq (Baird *et al.* 2008) and related methods have enabled genotyping of thousands of single nucleotide polymorphisms (SNPs), greatly extending our capacity to detect recent, fine-scale genomic divergences in non-model organisms (Andrews *et al.* 2016). Such genomic data have proven valuable for clarifying population dynamics and re-assessing species limits in taxa that have been historically difficult to characterize using other approaches, either due to recent diversification, morphological ambiguity, historical introgression, or some combination of these factors (Hohenlohe *et al.* 2013; Wagner*et al.* 2013; Escudero *et al.* 2014; Vargas *et al.*2017; Abdelkrim *et al.* 2018; Hinojosa *et al.* 2019; Hundsdoerfer *et al.* 2019). Alongside genomic advances, continued development of ecological niche models (ENMs) has facilitated an integrative approach to inferring processes that contribute to ecological diversification and reinforcement of species (Schluter 2001, 2009; Manel *et al.* 2003; Balkenhol *et al.* 2016). ENMs, generally parameterized by relating species' occurrences to geographic and environmental factors, may be used to quantify ecological niches, habitat associations, and potential geographic distributions of single species (Austin 1985; Peterson 2001; Guisan & Zimmermann 2000; Elith*et al.* 2006, 2011; Phillips *et al* . 2006; Zimmermann et al. 2010). However, ENMs may also be used in a comparative framework to assess niche divergence and conservatism among recently diverged evolutionary lineages, often delineated on the basis of genetic data (Sites & Marshall 2003; Graham *et al.* 2004, Kozak & Wiens 2006, Bond & Stockman 2008, Jezkova *et al.* 2009, Loera *et al.*2012; Newton *et al.* 2020).

The butterfly genus *Speyeria* Scudder, 1872 (Lepidoptera: Nymphalidae) is well known for its phenotypic variability and ambiguous evolutionary relationships among component lineages (dos Passos & Grey 1947; Moeck 1975; Dunford 2009). A total of 16 species are currently recognized in *Speyeria* , as well as over 110 morphologically variable subspecies and several species complexes with poorly understood evolutionary relationships (dos Passos & Grey 1947; Scott *et al.*1998; Dunford 2009; Pelham 2019). Among these, *S. hesperis*(Edwards, 1864) and *S. atlantis* (Edwards, 1862) form a large complex containing 26 subspecies (Pelham 2019). These include five subspecies in *S. atlantis* that are broadly distributed in conifer woodlands across North America from the Rocky Mountains to Newfoundland. More variation is taxonomically recognized in *S. hesperis* , which has 21 subspecies occurring in drier meadows and open forests throughout western North America and east to South Dakota and southeastern Manitoba (Pelham 2019). The two species contact each other in mixed forest areas from Manitoba to British Columbia and south along the Rocky Mountains to Colorado, exhibiting substantial morphological similarity between species in some areas as well as variation within species (dos Passos & Grey 1947; Moeck 1975; Dunford 2009).

Some taxonomic treatments have considered *S. hesperis* to be a subspecies of *S. atlantis* based on overall morphological similarity (Grey 1951; Miller and Brown 1981; Hammond *et al.*2013). Recent work recognizes these taxa as distinct species, based on assessments of morphological and genetic divergence, as well as an apparent lack of hybridization between sympatric populations (Campbell*et al.* 2017; de Moya *et al.* 2017; Campbell *et al.*2019; Riva *et al.* 2019; Thompson *et al.* 2019). However, the taxa remain difficult to reliably identify using morphology alone (Scott *et al.* 1998; Opler & Warren 2005). Additionally, Campbell*et al.* (2019) have recently shown, based on a limited number of specimens, substantial genomic divergence in SNPs between *S. hesperis* populations occurring north and east of the Rocky Mountains (throughout British Columbia, Alberta, and Saskatchewan in Canada and Montana and South Dakota in the United States; hereafter referred to as the "northern lineage"), and those occurring throughout the southwestern

and Great Basin regions of the United States ("southern lineage"). Further phylogenetic complexity is provided by interactions with species that have not historically been considered part of this complex, including intermediates between *S. hesperis* and *S. zerene* (Boisduval, 1852) (Campbell *et al.* 2019). This may have important implications for conservation initiatives for *S. zerene* , which has multiple subspecies experiencing significant population declines in western regions where *S. hesperis* and*S. zerene* co-occur (McHugh *et al.* 2013; Sims 2017). While these genetic assessments have helped clarify some taxonomic ambiguities, there have been no attempts to assess whether genomic divergences correspond to variation in ecological niches and habitat associations.

Our objective is thus to provide a proof-of-concept for the use of ecological modelling to strengthen genomic assessments of species boundaries and to clarify some of the extrinsic factors involved in the diversification of the *S. atlantis-hesperis* species complex. We use *de novo* SNPs to recover distinct genetic clusters of populations that maintain their genomic integrity in regions of contact (Sperling 2003), testing alternate hypotheses on species delimitation and phylogeographic factors that may have contributed to recovered genetic patterns. We additionally use ENMs to compare ecological niches of evolutionary lineages identified on a genomic basis to infer whether ecologically-based divergent selection is a likely contributor to their speciation and reinforcement of genomic integrity. Our integration of these methods demonstrates broad utility for the reconciliation of species concepts, such as the genomic integrity (Sperling 2003) and ecological species concepts (Van Valen 1976; Andersson 1990; Nosil 2012), that should contribute to stable species delimitations.

Methods

Specimen collection and identification

Specimens were collected using aerial nets and were either preserved in ethanol or frozen at -20°C until DNA was extracted from each sample. Following Campbell *et al.* (2019), morphological identifications to subspecies were made using multiple sources, including range information (Moeck 1975), field markings (Bird *et al.* 1995; Brock & Kaufman 2003; Dunford 2009; Warren *et al.* 2012), and comparison to specimens in the Bean Museum collection at Brigham Young University or the personal reference collection of E. Gage. In addition to specimens of *S. atlantis* and *S. hesperis* , we included several specimens of *S. zerene* to test for both ancient and contemporary admixture, since SNP-based Structure analysis by Campbell*et al.* (2019) indicated limited putative hybridization between*S. zerene* and *S. hesperis* , and phylogenetic analyses additionally showed mito-nuclear discordance in relationships among*S. hesperis, S. atlantis,* and *S. zerene* . The total dataset was comprised of 113 specimens in 14 subspecies of *S. hesperis,*19 specimens in three subspecies of *S. atlantis,* 18 specimens in four subspecies of *S. zerene* , and for phylogenetic analysis, two outgroup specimens of *S. cybele cybele* (Fabricius, 1775) (Table S1).

Molecular data generation and processing

DNA extraction, mitochondrial *COI* gene amplification, ddRAD (Peterson *et al.* 2012) and two-enzyme GBS (Poland *et al.*2012) library preparation and sequencing, and initial mitochondrial and SNP data processing follows Campbell *et al.* (2017, 2019) and so are not described in detail here. SNPs were genotyped *de novo*using the Stacks v. 2.3 pipeline (Catchen *et al.* 2011; Rochette*et al.* 2019) with default parameter settings except for the following: the *n* parameter, which controls the number of mismatches tolerated per locus during catalog construction in*cstacks* , was set to 2 instead of 1; we only retained loci that were found in 80% of any single population (the "r80" principle of Paris *et al.* 2017); and a single, random SNP from each locus was output during final processing in the *populations* program of Stacks to reduce genomic linkage. We conducted additional SNP filtering in vcftools 0.1.14 (Danecek *et al.* 2011) to retain only loci with a minimum minor allele frequency of 3% and to further reduce the global missing data per locus to a maximum of 20%.

Phylogenetic and population genetic analyses

Phylogenetic analyses for both the *COI* gene and the filtered genomic SNPs were conducted in IQ-TREE

1.3.10 (Nguyen *et al.*2015). Model testing, SH-aLRT branch testing, and 1000 replicates of ultrafast bootstrapping (Hoang *et al.* 2018) were conducted in the program.

Mitochondrial *COI* gene data was used to build a minimum spanning haplotype network (Bandelt *et al.* 1999) in the program PopART (Leigh & Bryant 2015), which outputs a visual representation of the population genetic relationships between *COI* haplotypes. We used Structure version 2.3.4 (Pritchard *et al.* 2000) and TESS version 2.3.1 (Chen *et al.* 2007) to infer population structure in the SNP dataset. While both programs take a similar Bayesian approach to population clustering based on changes in allele frequency, TESS differs from Structure by additionally incorporating a spatial component for inferring genetically disparate populations that may result from geographic discontinuities. This is particularly useful when genetic structure correlates to isolation by distance, which can contribute to population over-splitting in non-spatial programs (Chen *et al.*2007). Campbell *et al.* (2019) showed strong genetic sub-structuring within *S. hesperis* that corresponded to sets of populations sampled southwest and northeast of the Rocky Mountains; TESS and Structure were compared to clarify the extent that geography influenced these results.

Structure analyses were run using the admixture model without using sampling locations as a prior. We tested K = 1-10 with a burn-in period of 150,000 generations, 750,000 MCMC chains, and 10 replicate runs for each K value. We also conducted separate substructure analyses for *S. zerene, S. atlantis,* and the northern cluster of *S. hesperis* , testing K = 1-5 for each. We used CLUMPAK v. 1.1 (Kopelman *et al.* 2015) to average runs and determine the optimal K considering both ΔK (Evanno*et al.* 2005) and LnPr(K) (Pritchard *et al.* 2000). We used TESS to infer K = 2-7, and incorporated weights on the Voronoi network by computing pairwise Euclidean distances between the geographic coordinates for each specimen. These weights correct for regions with irregular or unequal sampling during Voronoi neighbourhood estimation. We ran this analysis using the CAR admixture model (Durand*et al.* 2009) for 10 replicates per K , with a burn-in period of 50,000 and 200,000 sweeps (analogous to "generations" in Structure), and sampled the spatial interaction parameter and variance during the MCMC runs. Following program recommendations, we averaged the Deviance Information Criterion (DIC) score for the 10 runs of each value of K , and then identified the optimal K as the lowest value of K at which the DIC scores stabilized.

Species delimitation and introgression analyses

We conducted species delimitation testing using BFD* (Leaché *et al.* 2014) implemented in the SNAPP plug-in (Bryant *et al.* 2012) for BEAST 2 (Bouckaert *et al.* 2014). SNAPP uses the multispecies coalescent (MSC) to estimate trees, effective population sizes, and divergence times from SNPs by inferring probabilities of allele frequency change, and then outputs a posterior distribution that represents different estimations of the species tree (Bryant *et al.* 2012). BFD* outputs marginal likelihood estimations (MLE) for each species delimitation model, which are used to calculate Bayes Factors (Grummer *et al.* 2014) and determine the best supported model (Leaché *et al.* 2014). Because this program is computationally demanding, we reduced the total number of individuals to 27 to speed up the analysis. Using the K = 6 Structure analysis as a guide (results described below), we included between three to six specimens from major genetic clusters as follows: six each from *S. zerene*(sampled from Alberta, Idaho, Nevada, and California) and *S. atlantis* (sampled from Alberta, Ontario, and Colorado)*,* five from the northern cluster of *S. hesperis* (sampled from Alberta, British Columbia, Montana, Colorado, and South Dakota)*,* and, for the southern clusters of *S. hesperis* , we sampled four specimens from the New Mexico and Arizona population, and three specimens each from the southern Utah population and the central population (sampled from Idaho, Colorado, and Utah). We chose individuals for the BFD* analysis that had little or no genomic admixture in the Structure and TESS results to ensure that these analyses weren't biased by contemporary hybridization.

We tested five species delimitation models of *S. atlantis, S. hesperis,* and *S. zerene,* informed by existing species delimitations and alternate assignments recovered in the clustering and phylogenetic analyses: (i) the "*a priori* " model, following the current species delimitation for *S. zerene, S. hesperis,* and*S. atlantis* ; (ii) the "2 species" model, following the K= 2 Structure results; (iii) the "4 species" model, which splits*S. hesperis* into northern and southern species; (iv) the "3 species" model, which lumps northern *S. hesperis* and *S. atlantis* ; and (v) the "6 species" model, following the K = 6 Structure and TESS results.

Following BFD* recommendations, we set the mutation parameters $u$and v to 1, and allowed the coalescence rate to be sampled via MCMC to reflect probable differences in population size between lineages. We also included non-polymorphic sites in the analysis because our dataset contained some missing data. BFD* implements a birth-only Yule tree prior, which we set to have a gamma distribution with a single parameter, λ, governing speciation rate (Leaché *et al.* 2014). We calculated λ from the maximum likelihood consensus SNP tree output from IQ-TREE using the package phytools 0.6-99 (Revell 2012) implemented in R 3.6.1 (R Core Team 2017), and used it to determine the β scale parameter with an α shape parameter of 2. This gave us a gamma distribution of λ=65.96, α=2, and β=32.9. To avoid potential sampling bias by the program due to the narrow parameter distribution for λ and β indicated by the data, we further relaxed our λ to 200 and our β to 100. Following program recommendations, we set our rate priors to also follow a gamma distribution, with λ=10, α=1, and β=250 (Leaché and Bouckaert 2018), and ran each scenario with 1 million MCMC chains, 200,000 burn-in generations, and 24 path sampling steps. Convergence was assessed using Tracer v. 1.6.0 (Rambaut *et al.* 2018), and TreeAnnotator 2.4.7 (Drummond & Rambaut 2007) was used to generate the maximum clade credibility tree. We additionally used DensiTree v. 2.0.1 (Bouckaert 2010) to visualize topological discordance in the posterior distribution of trees recovered during BFD* model testing.

We used TreeMix (Pickrell & Pritchard 2012) to assess putative introgression between *S. zerene, S. hesperis,* and *S. atlantis* . TreeMix uses a Gaussian model to estimate drift between populations, and identifies population pairs that have the highest residuals, which are interpreted by the program as putatively experiencing gene flow. TreeMix subsequently plots migration edges and infers directionality of admixture between these populations in order to improve the likelihood score of the model (Pickrell & Pritchard 2012; Kozak *et al.* 2018). We built an unrooted maximum likelihood phylogeny with 100 bootstrap replicates using blocks of 50 SNPs, and then sequentially added 0-5 migration edges to the tree. For each model, we re-ran TreeMix three times to ensure consistency in our results, and used jackknifing to estimate the weight and significance of each migration edge. We additionally calculated f 3 statistics for all possible combinations of populations to substantiate plotted migration edges using the *threepop* command implemented in TreeMix.f 3 tests estimate admixture between triplets of specified parental and mixed populations; a significantly negative f 3 statistic supports the hypothesis of an introgression event between two parental populations and a putatively admixed population (Reich *et al.* 2009; Pickrell & Pritchard 2012).

Ultimately, support for migration events between populations of *S. hesperis, S. atlantis,* and *S. zerene* was determined by considering multiple lines of evidence, including an assessment of the residuals for each model, the statistical significance of putative migration events, and concordance between the migration edges plotted on the maximum likelihood tree and admixture events indicated by the$f$ 3 statistics.

Ecological niche divergence estimation of S. atlantis *and*S. hesperis

We created a series of ecological niche models (ENMs) to infer whether three major genomic lineages identified in this study, *S. atlantis* , northern *S. hesperis* , and southern *S. hesperis* , satisfy criteria for classification as distinct ecological species (*sensu* Nosil 2012) . ENMs were fit using MaxEnt software (Phillips *et al* . 2006), which uses machine-learning maximum entropy modelling of presence-only data to quantify species' ecological niches as well as predict habitat associations and potential geographic distributions across heterogeneous landscapes (Elith *et al.* 2006, 2011). For each of the three lineages, inputs for ENMs included the georeferenced localities of sequenced individuals and a set of geographic information systems (GIS) predictor variables, classified as either geographic or environmental; derivation and sources of the predictor variables used in these analyses are provided in Supplemental File 1.

Before fitting ENMs, we generated buffered (100 km) minimum convex polygons around the localities of sequenced individuals assigned to each of the three lineages. GIS data layers were clipped to these polygons and 10,000 background points were generated within polygons to sample available habitat. For each lineage, we removed duplicate locality coordinates and generated five different locality lists, each withholding a different 20% of the data to allow for K -fold analysis. For each of these five lists, the remaining 80% of localities were used to fit an ENM (via the R package dismo, Hijmans *et al.*2011) and the withheld 20% were used to evaluate its predictive power. Model evaluation was completed using receiver operating characteristic

(ROC) analysis and area-under-the-curve (AUC) scores (Phillips *et al.* 2006). AUC scores are bound between 0 and 1, with higher values indicating greater predictive power. Background points (n=10,000) used in model evaluation were confined to each lineage's buffered minimum convex polygon, meaning only habitat within polygons was defined as available in AUC estimation to avoid inflated estimates of the models' predictive power. Following model evaluation, fitted ENMs for each lineage were used to predict habitat suitability across the entire study area. Each 1-km grid cell received a habitat suitability score ranging from 0 – 1, with higher values indicating higher suitability.

Niche divergence among genomic lineages was quantified in a pairwise fashion. For each pair of lineages, we assessed power with which one lineage's set of ENMs predicted the localities of the other lineage within its respective buffered minimum convex polygon. To accomplish this, ENMs for one lineage were used as fitted models and the other lineage's localities were used as validation data for estimation of "between-lineage" AUC scores. Background points (n =10,000) used in these model evaluations were confined to the buffered minimum convex polygon of the validation data. Within- and between-lineage AUC scores for each pair of lineages were then used as dependent variables in generalized linear mixed effects models, fit using a beta distribution and a logit link using the R package glmmTMB (Brooks*et al.* 2017). Within linear mixed effects models, a "within-*vs.* between-lineage" binary predictor variable reflected whether each AUC score corresponded to a within-lineage (0) or between-lineage (1) model evaluation. For pairs of lineages, these models thereby quantified whether there was a significant reduction in the power of one lineage's ENMs when predicting the other lineage's localities. To control for nonindependence resulting from partially overlapping sets of localities used to fit each lineage's five ENMs, the ID of the lineage for which each ENM was fitted was included as a random effect. Within each linear mixed effects model, a significant negative effect of the "within- *vs.* between-lineage" binary variable would indicate that predicted habitat suitability of the two lineages involved in the pairwise comparison are significantly different, suggesting significant niche divergence.

It is possible that spurious inferences of niche divergence may arise from differences in available habitat used to parameterize ENMs. To investigate this possibility, we built a series of null ENMs for each lineage using randomly generated localities confined to the buffered minimum convex polygons of the actual localities of sequenced individuals (1 point/10,000 km^2 for each lineage).K -fold analysis was completed as described above using five different random locality lists, each withholding a different 20% of the random localities. We then repeated within- and between-lineage model evaluations for each pair of lineages. Within- and between-lineage AUC scores were again compared using linear mixed effects models, identical in structure to those described above. Within each linear mixed effects model, the absence of a significant negative effect of the "within- *vs.* between-lineage" binary variable would indicate that observed niche divergences cannot be attributed to biases arising from differences in available habitat.

Assessment of barriers to gene flow in S. hesperis

The northern and southern *S. hesperis* lineages identified in this study are largely parapatric in their distributions. It is therefore possible that genetic drift resulting from barriers to dispersal (i.e., vicariance and speciation without selection or mutation-order speciation), rather than ecologically-based divergent selection (i.e., ecological speciation), has been the dominant evolutionary process underlying diversification and reinforcement of the identified lineages (*sensu* Nosil, 2012). To test for this possibility, we assessed whether there are present-day barriers to dispersal between the northern and southern *S. hesperis* lineages related to arrangements of suitable habitat. This was accomplished using resistance surfaces parameterized as the inverse of predicted habitat suitability (McRae & Beier 2007; Wang et al. 2008; Storfer et al. 2010; Wang et al. 2012; MacDonald et al. 2020). This approach assumes that organisms are more likely to disperse within suitable habitat and experience high resistance when moving through unsuitable habitat; large stretches of unsuitable habitat thereby pose significant barriers to dispersal (Coyne & Orr 2004; Crispo et al. 2006; McRae 2006; McRae & Beier 2007; Thorpe et al. 2008, 2010; Sánchez-Ramírez et al. 2018). We averaged resistance surfaces of the northern and southern *S. hesperis* lineages to generate a single resistance surface reflecting the probability that dispersing individuals of the two lineages will come into contact that could result in reciprocal gene flow. Using this single resistance surface, we then calculated pairwise resistance

distances between all individuals using the R package gdistance (van Etten 2017) and organized them into a pairwise matrix. These resistance distances are analogous to circuit distances (McRae & Beier 2007), measuring expected random-walk commute time between nodes (i.e., localities of sequenced individuals) in a graph (i.e., resistance surface) (Chandra et al. 1996). We also generated a pairwise matrix of Euclidean distances between all individuals using the R package sp (Pebesma & Bivand 2005). A third pairwise matrix ("lineage distance") indicated whether individuals belonged to the same lineage (value = 0) or to different lineages (value = 1).

We used a partial mantel test to evaluate whether resistance distances between individuals of different lineages were significantly greater than those between individuals of the same lineage after controlling for Euclidean distance. This analysis effectively evaluates whether significant barriers to dispersal exist between the northern and southern *S. hesperis* lineages. A significant correlation between resistance distance and lineage distance after partialling out Euclidean distance would suggest that reduced gene flow resulting from barriers to dispersal (vicariance) cannot be ruled out as a principal mechanism reinforcing the genomic integrity of the northern and southern *S. hesperis* lineages. Alternatively, the absence of a significant correlation between resistance distance and lineage distance after partialling out Euclidean distance would suggest that ecologically-based divergent selection likely contributes to reinforcement of the northern and southern *S. hesperis* lineages.

Results

Dataset construction and phylogenetic analyses

After filtering, our SNP dataset contained 1026 SNPs (min. locus depth: 8, max. locus depth: 204, mean locus depth: 65.6). The *COI* dataset consisted of 648 sites (577 invariant and 71 variant), 58 of which were phylogenetically informative.

The SNP species tree shows *S. cybele* , *S. atlantis* , and *S. zerene* as monophyletic clades (Fig. 1a), but indicates a polyphyletic relationship for the northern and southern *S. hesperis* lineages. The northern lineage was additionally paraphyletic with *S. atlantis,* and contained subspecies *S. h. beani* (Barnes & Benjamin, 1926) and *S. h. dennisi* dos Passos & Grey, 1945 from Alberta, *S. h. hutchinsi* dos Passos & Grey, 1947 from Montana, *S. h. brico* Kondla, Scott & Spomer, 1998 and *S. h. beani* from British Columbia, *S. h. lurana* dos Passos & Grey, 1945 from South Dakota, *S. h. ratonensis* Scott, 1981 from southeastern Colorado, and *S. h. irene* (Boisduval, 1869) from California, but did not exhibit consistent geographic sub-clustering. The southern *S. hesperis* lineage was sister to *S. zerene* , and itself had two major geographic groupings. A large grade contained *S. h. tetonia* dos Passos & Grey, 1945 sampled in northern Utah and southern Montana, *S. h. chitone* (Edwards, 1879) sampled in southeastern Utah, *S. h. electa* (Edwards, 1878) sampled in southwestern Colorado, and *S. h. viola* dos Passos & Grey 1945 from Idaho. A monophyletic clade contained *S. hesperis* from New Mexico and Arizona, and broadly separated a southern New Mexican population of *S. h. capitanensis* Holland, 1988 sampled in the Sacramento Mountains from the more northern population of *S. h. dorothea* Moeck, 1947 sampled in the Sandia Mountains of New Mexico, which clustered with *S. h. nausicaa* (Edwards, 1874) from Arizona. Phylogenetic comparison of the SNP and *COI* datasets recovered extensive mito-nuclear discordance and a reduction in monophyly on the *COI* tree (Fig. 1b), largely consistent with Campbell *et al.* (2019).

Haplotype network, Structure, and TESS

Structure analysis of SNP data suggested two optimal values of K , indicating substructure in the data. ΔK supported $K = 2$ (Fig. S1), which grouped a broadly northern *S. hesperis* cluster (specimens from British Columbia, Alberta, Montana, South Dakota, California, and southeastern Colorado) with *S. atlantis,* and a southern *S. hesperis* cluster (specimens from Utah, Idaho, southern Montana, southwestern Colorado, New Mexico, and Arizona) with *S. zerene* (Fig. 2a). LnPr(K) supported $K = 6$ and ΔK additionally had a small peak at $K = 6$. This resolved *S. zerene, S. atlantis* , and northern *S. hesperis* as distinct clusters, and further separated southern *S. hesperis* into three geographically-defined clusters: 1. "central" (specimens from northern Utah, Idaho, southern Montana, and southwestern Colorado); 2. southern Utah; and 3. New

Mexico and Arizona. Two of the northern New Mexico *S. h. dorothea* specimens and all the Arizona*S. h. nausicaa* specimens appeared to be mixtures between the southern New Mexico and central populations. Similarly, the southwestern Colorado *S. h. electa* specimens were intermediate between the central and southern Utah populations, and the Idaho *S. h. viola*specimens were intermediate between the central and northern populations (Fig. 2b). Substructure analysis of *S. zerene* indicated genetic differences in *S. zerene zerene* from California that were not shared by any *S. zerene* specimens sampled from Nevada, Utah, Idaho, Montana and Alberta. Substructure analysis of the northern*S. hesperis* cluster did not indicate additional geographic substructuring; both LnPr(K) and ΔK indicated an optimal$K$ of 2, however this did not produce any meaningful sub-structure in the data that corresponded to sampling locality, and given that ΔK cannot estimate $K = 1$, we suggest that $K = 1$ is a more meaningful result.

TESS supported $K = 5$. This analysis was largely congruent with the $K = 6$ Structure results, except that it lumped the central and southern Utah populations into a single cluster (Fig. 2a).$K = 6$ had a similar DIC score to that of the $K = 5$ TESS results, but failed to add any meaningful geographic substructuring (Fig. S1), further supporting $K = 5$ as optimal for this analysis. Both TESS and Structure indicated a few likely hybrids: one *S. hesperis* sampled from southern Utah shared ancestry with the northern New Mexico *S. hesperis* population, our single specimen of*S. hesperis* sampled from California (putatively *S. h. irene*) was admixed with *S. zerene* , and one *S. zerene* from California shared ancestry with the southern Utah *S. hesperis*population.

The minimum spanning haplotype network depicted distinct *S. atlantis* and *S. hesperis* clusters, however there was very little haplotype variation within either *a priori* species (Fig. 2c). In all cases there were only one or two nucleotide differences between the "distinct" specimens and the major haplotype group for each species. For *S. atlantis,* this haplotype variation largely correlated to sampling location - eastern *S. atlantis canadensis* (dos Passos, 1935) specimens sampled in Ontario and Quebec and the *S. atlantis sorocko* Kondla & Spomer, 1998 specimens from Colorado were marginally distinct from *S. atlantis hollandi* (Chermock & Chermock, 1940) sampled in Alberta and Manitoba; one *S. atlantis hollandi* from Alberta and one from Manitoba were minimally different from the major "*hollandi* " haplotype. This geographic pattern was not observed in *S. hesperis,* and almost all the specimens sampled had identical haplotypes regardless of sampling location. Comparatively,*S. zerene* had much more haplotype diversity in the minimum spanning network, with three distinct haplogroups. One group consisted of the *S. zerene* specimens sampled from Alberta, Idaho, Montana, and Utah and was the most distinct from *S. hesperis* and *S. atlantis* , and a second group containing *S. zerene zerene* from California was intermediate between the *S. hesperis* and *S. atlantis* haplogroups. Interestingly, the third *S. zerene*haplotype that was found in *S. zerene gunderi* sampled from Nevada was identical to the major *S. hesperis* haplotype (Fig. 2c), but these individuals did not appear admixed with *S. hesperis* in the Structure or TESS analyses of SNPs; the single likely *S. zerene*hybrid was instead from California, and had a haplotype consistent with the rest of the Californian *S. zerene* specimens.

Species delimitation and introgression analyses

Of the five species models tested in BFD*, the "4 species" model (*S. zerene, S. atlantis,* northern *S. hesperis* , and southern *S. hesperis*) had the highest marginal likelihood estimate (MLE) and the most strongly negative Bayes Factor (BF), indicating that this model was the best supported by the data (Table 1). The maximum clade credibility (MCC) tree indicated strong support for the northern *S. hesperis* and *S. atlantis* clade (posterior probability of 1), but the clade containing *S. zerene* and southern *S. hesperis* was less supported with a posterior probability of 0.87 (Fig. 3a, left panel). The MCC tree additionally indicated that the southern *S. hesperis/S. zerene* clade diverged slightly earlier than the clade containing *S. atlantis* and northern *S. hesperis* .

DensiTree visualization of the BFD* results indicated discordance in the relationship between *S. zerene* and the remaining three species on the tree. The major recovered topology, which accounted for 87.4% of the sampled trees (shown in blue in Fig. 3a, right panel), was the same as the topology presented in the MCC tree, however the second most common topology (7.1% of the sampled trees, shown in purple) depicted*S. zerene* as basal to the other three species. A third topology accounting for the remaining 5.4% of the sampled

trees (shown in orange) depicted *S. zerene* as the sister taxon to the *S. atlantis/* northern *S. hesperis* clade. The *S. atlantis* /northern *S. hesperis* sister relationship was consistently recovered in each sampled topology.

Given the discordant relationship between *S. zerene* and *S. atlantis/S. hesperis* in our phylogenetic and BFD* analyses, we used TreeMix to test for introgression between these species. The unrooted maximum likelihood phylogeny produced in TreeMix (Fig. 3b, Fig. S2) was largely consistent with the SNP phylogeny in Fig. 1. Heatmaps containing the pairwise population residuals for each migration model generally indicated improved fit with the addition of migration events (Fig. S2), but this seemed to plateau for models m 3-5; the residual plots for three, four, and five migration events were highly similar, suggesting that continued addition of migration events past this point did not greatly improve fit. The m 3 model indicated a substantial migration event from the interior of the branch containing *S. zerene* to the central *S. hesperis* population (Fig. 3b; migration weight = 0.41, p = 0); a migration edge plotted along the interior of the branch rather than the terminus may indicate historical admixture or admixture from unsampled populations (Pickrell & Pritchard 2012). This model also indicated two less substantial, but still statistically significant, migration events between *S. zerene* and the clade containing the central and New Mexico/Arizona *S. hesperis*populations (migration weight = 0.07, $p = 0.037$), and between*S. zerene* and *S. atlantis* (migration weight = 0.07,$p = 0.017$). Though models m 4 and m 5 had similar residuals (Fig. S2), they indicated non-significant migration weights between some populations: in m 4 the migration edge between the New Mexico/Arizona *S. hesperis* population and *S. atlantis*was non-significant (migration weight: 0.02, $p = 0.169$), and in$m$ 5 the edges between *S. zerene* and the clade containing the New Mexico/Arizona and central *S. hesperis* populations (migration weight = 0.09, $p = 0.058$), between the New Mexico/Arizona *S. hesperis* populations and *S. atlantis*(migration weight = 0.02, $p = 0.092$), and between the central and northern *S. hesperis* populations (migration weight = 0.01,$p = 0.345$) were non-significant.

Of the 61 f 3 tests for introgression that we computed, only three were significant (Table S2); one indicated admixture from *S. zerene* and the southern Utah population of *S. hesperis* into the central *S. hesperis* population ($p = 0$), and another from*S. zerene* and the New Mexico/Arizona population of *S. hesperis* into the central *S. hesperis* population ($p = 0.003$). The third test suggested gene flow from the New Mexico/Arizona and northern *S. hesperis* populations into the central *S. hesperis* population ($p = 0.035$).

Ecological niche divergence

ENMs sufficiently predicted habitat suitability within each lineage's buffered minimum convex polygon, as indicated by mean AUC scores:*S. atlantis* (mean = 0.809, s.e. = 0.084), northern *S. hesperis* (mean = 0.810, s.e. = 0.063), and southern *S. hesperis*(mean = 0.803, s.e. = 0.085) (Fig. 4). Visual inspection of predicted habitat suitability surfaces across the entire study landscape suggests that the three genomic lineages are divergent in their respective habitat associations and ecological niches, and that the highest density of suitable habitat for each lineage is generally found within and adjacent to their buffered minimum convex polygon. Relative contributions of geographic and environmental predictor variables to ENMs, measured as the drop in AUC scores after each variable was randomly permuted, are reported in Table 2. For each of the three lineages, land cover and growing degree days had the greatest contribution to ENMs. Contributions of other variables varied considerably among lineages.

Linear mixed effects models indicated there was a significant reduction in the power of each lineage's ENMs when predicting the localities of each of the other two lineages. The coefficient of the "within-*vs.* between-lineage" binary predictor variable was significantly negative for each pairwise comparison: *S. atlantis* and northern*S. hesperis* (β = -0.292; $p = 0.028$), *S. atlantis*and southern *S. hesperis* (β = -0.6811; $p < 0.00$), and northern and southern *S. hesperis* (β = -0.333;$p = 0.023$). Together, these results indicate that all three lineages are significantly divergent in their habitat associations and ecological niches.

These analyses were repeated using null ENMs built with randomly generated localities confined to each lineage's buffered minimum convex polygon. Linear mixed effects models addressing resulting AUC scores indicated no significant reduction in the power of each lineage's null ENMs when predicting the random localities generated for the other lineages. Specifically, the coefficient of the "within- *vs.*between-lineage"

binary predictor variable was non-significant for *S. atlantis* and northern *S. hesperis* (β = -0.012; p= 0.822), *S. atlantis* and southern *S. hesperis* (β = 0.020;p = 0.751), and northern and southern *S. hesperis* (β = 0.015; p = 0.810). These results indicate that observed differences in habitat associations and ecological niches cannot be attributed to biases arising from differences in available habitat among the three lineages.

Assessment of barriers to gene flow in S. hesperis

A partial mantel test indicated that lineage distance (same vs. different lineage) was not significantly related to resistance distance (r = -0.111; p = 0.987) after partialling out the relationship between lineage distance and Euclidean distance. This result suggests that, although dispersing individuals of each lineage are less likely to contact individuals of the other lineage, this is best attributed to the geographic separation of the ranges of the lineages (i.e., parapatry) and not to arrangements of suitable habitat that might present significant barriers to dispersal.

Discussion

Species delimitations that are informed by genetic data have undergone a renaissance as increasingly more sophisticated sequencing technology and analytical methods have become available. But genes are only part of what makes a species. Species delimitations informed by ecological characteristics – the phenotypic interactions of individuals with their environment – have been difficult to incorporate quantitatively and are usually relegated to natural history accounts. Our study uses a notoriously confusing species group of butterflies to demonstrate an analytical integration of genetic and ecological species concepts. This approach supports species delimitations that should ultimately be more stable and meaningful in conservation contexts and citizen science.

Evidence for historical introgression between S. hesperis *and* S. zerene

Our genomic analyses suggest a complex, shared evolutionary history between *S. atlantis, S. hesperis,* and *S. zerene* . BFD* species delimitation supported separation of northern and southern *S. hesperis* lineages as distinct species, and a sister relationship between *S. atlantis* and northern *S. hesperis* , consistent with our SNP phylogeny (Fig. 1a). However, the relationship of *S. zerene* to the other clades varied. This is interesting, as recent molecular phylogenetic work on *Speyeria* generally indicates a non-sister relationship between *S. zerene* and *S. hesperis* / *S. atlantis* (de Moya *et al.* 2017; Campbell *et al.* 2017, 2019; Thompson *et al.* 2019). It is likely that the recovered polyphyly of *S. hesperis* and the sister relationship between southern *S. hesperis* and *S. zerene* presented here is partly due to the omission of other *Speyeria* species in our phylogenetic analyses, but also due to probable introgression between *S. hesperis* and *S. zerene,* which is more explicitly indicated by SNP-based admixture and *COI* haplotype analyses (Fig. 2, Fig. 3, Table S2).

While the results of our SNP-based analyses and the lack of sequence variation in the mitochondrial haplotype shared between *S. zerene* and *S. hesperis* support a hypothesis of introgression between these non-sister species, they do not sufficiently clarify the direction of gene flow between them; TreeMix and f 3 tests indicated introgression from *S. zerene* into *S. hesperis* , however our inference that the *S. hesperis* haplotype occurs throughout the entire *S. hesperis* sampled range, but only in the Nevadan part of the range of *S. zerene,* suggests that it originated in *S. hesperis* . Expanded sampling of both species is needed to clarify the pervasiveness of this haplotype in *S. zerene* and to validate its origin.

In contrast to mtDNA, nuclear SNPs across the range of *S. hesperis* do not show the same obvious reduction in genetic variability, suggesting that a strong selective sweep leading to a severe bottleneck event has recently caused the loss of other variable mitochondrial haplotypes (Sonsthagen *et al.* 2017; Hurst & Jiggins 2005). A candidate for facilitating such a process is *Wolbachia* Hertig & Wolbach, 1924, maternally-inherited, endosymbiotic bacteria that can facilitate the spread of particular mitochondrial haplotypes throughout populations and species, reducing haplotype variation (Werren *et al.* 2008; Kodandaramaiah *et al.* 2013; Ahmed *et al.* 2015). *Wolbachia* infections have been reported in several *Speyeria* species, including *S. zerene* (McHugh *et al.* 2013), but not yet within the *S. atlantis-hesperis* complex (Hamm *et al.* 2014). *Wolbachia* infection via introgression offers a plausible and testable hypothesis that could explain the observed haplotype

sharing between Nevadan *S. zerene gunderi* and*S. hesperis* in the absence of contemporary nuclear admixture in the sampled specimens, which may further clarify the historical relationship between these taxa.

Evidence of niche divergence

Comparison of ENMs indicated that the *S. atlantis,* northern*S. hesperis* , and southern *S. hesperis* evolutionary lineages, delineated on the basis of genomic data, significantly differ in their habitat associations and ecological niches. The relative magnitude of the "within- *vs.* between-lineage" coefficient estimate may be interpreted not only as a measure of the relative strength of niche divergence, but also as an indicator of whether or not genomic divergences likely reflect ecological speciation events. Comparison of these coefficient estimates across models suggest that niche divergence was greatest between *S. atlantis* vs. southern*S. hesperis* , followed by northern *S. hesperis* vs. southern*S. hesperis* and *S. atlantis* vs. northern *S. hesperis* , which were approximately equivalent in their strength of pairwise niche divergence. This ordering corresponds to that of genomic divergences estimated between *S. atlantis* and northern and southern *S. hesperis* (Fig. 1, Fig. 2, Fig. 3). Although degree of divergences between pairs of lineages appears to approximately correspond to degree of geographic separation, null ENMs built from random localities indicate that observed niche divergences cannot be attributed to differences in available habitat between lineages (spurious niche divergence). Furthermore, although northern and southern*S. hesperis* are largely allopatric in their distributions, our resistance-based analyses did not detect any significant barriers to dispersal between them, conferring further support to the hypothesis that ecologically-based divergent selection has been the principal process contributing to diversification and reinforcement of the lineages' genomic integrity, rather than speciation without selection or mutation-order speciation associated with barriers to dispersal (i.e., vicariance). We therefore suggest that present-day parapatry between northern and southern *S. hesperis* is a consequence, rather than cause, of ecologically-based divergent selection, as projections of suitable habitat across the entire study landscape demonstrate clear geographically-structured divergence in habitat associations between the lineages (Fig. 4).

Assessment of species limits in S. hesperis and *S. atlantis*

Our results consistently show that the northern *S. hesperis*lineage is more genetically similar to *S. atlantis* than to any genetic clusters within the southern *S. hesperis* lineage, but we did not recover any evidence of admixture between *S. atlantis* and northern *S. hesperis,* despite sampling both species in sympatric portions of their ranges (mostly in Alberta, Canada); these results are consistent with other genetic surveys (Thompson *et al.* 2019; Campbell *et al.* 2019). Past work has noted differences in micro-habitat preference between *S. atlantis* and *S. hesperis* in regions where they co-occur (Bird *et al.* 1995; Guppy & Shepard 2001; Dunford 2009; Riva *et al.* 2019), and the results of our ENMs indicate that divergences in habitat associations are likely significant enough to limit gene flow between species, even in sympatric regions. Our results also indicate relatively little mitochondrial and SNP differentiation between the subspecies *S. atlantis hollandi, S. a. sorocko,* and *S. a. canadensis* relative to the other taxa in this study. Thus, *S. atlantis* is an independently evolving lineage distinct from *S. hesperis* , and recognition of the species status of *S. atlantis* should be maintained.

BFD* species delimitation using SNPs indicated clear support for splitting northern and southern *S. hesperis* into distinct species (Fig. 3a). Both Structure and TESS failed to recover any substructure in northern *S. hesperis* despite broad geographic sampling, and TreeMix and f 3 analyses indicated probable gene flow between northern *S. hesperis,* southern *S. hesperis,* and *S. zerene,* particularly in or near the central population of southern*S. hesperis* (Fig. 2, Table S2). This is also the approximate boundary between the sampled northern and southern *S. hesperis*lineages, so it is possible that northern *S. hesperis* expanded poleward from this geographic region into newly available habitat following the last glacial retreat, and that these lineages may have initially continued to exchange genes with each other and *S. zerene* . If this hypothesis is accurate, then the lack of recovered population structure in northern *S. hesperis* may be partly reflective of this recent range expansion, as well as due to few barriers to dispersal and gene flow in the relatively homogenous prairie/open forest habitat of northern *S. hesperis* compared to the more disjunct habitat of southern *S. hesperis* in the southern Rocky Mountains. The haplotype sharing between *S. zerene*

and northern and southern *S. hesperis* further suggests that introgression between these species occurred prior to this range expansion, and emphasizes that *COI* is only partially informative for clarifying species boundaries in this complex. ENMs similarly suggest that ecologically-based divergent selection is likely a principal process underlying divergence and reinforcement of the northern and southern lineages, even in the absence of barriers to dispersal.

Taxonomic revision to S. hesperis

Our results unambiguously support recent evolutionary and ecological divergences between northern and southern lineages of *S. hesperis* , but also indicate that these lineages currently exist as distinct entities shaped by interactions with other species and the environment that nonetheless maintain their genomic integrity. We therefore recommend that the two lineages should be recognized as distinct species. Following taxonomic priority (Pelham 2019), the northern lineage should continue to be referred to as *S. hesperis* , and the oldest southern subspecies name sampled in this study, *S. h. nausicaa* , should be elevated to species level,*S. nausicaa* , to represent the entire southern lineage. This taxonomic revision remains a hypothesis that requires further sampling and genomic assessment, particularly in California and the Great Basin, where lineages that diverged before the Pleistocene may persist. We also detected introgression between *S. zerene* and *S. hesperis/S. nausicaa* that may be a source of nuclear gene tree-species tree discordance. This should be pursued due to the conservation status of several *S. zerene* subspecies.

Conclusions

Our results substantiate species-level genomic divergences between*S. atlantis* , *S. hesperis* , and *S. nausicaa* , and analyses using ENMs and landscape resistance surfaces have enabled us to attribute the maintenance of genomic integrity in each of these lineages to a likely mechanism - ecologically-based divergent selection. In addition to our results, we suggest that there is great opportunity to build on this approach in subsequent systematic work. In particular, the incorporation of ecological modelling in genetic species delimitations may be used to reciprocally inform Bayesian priors for phylogeographic and multispecies coalescent-based modelling. Such an integration of genetic and ecological analyses should be particularly useful for species delimitations that have conservation impacts or high visibility in citizen science, and could be readily integrated into a conservation framework that links genomic divergence to ecological distinctiveness, informing prioritization of habitat and biodiversity management. This multidisciplinary method of species delimitation is thus positioned to contribute to stable taxonomies, delimit meaningful biodiversity units for conservation, and characterize extrinsic factors that influence patterns of lineage diversification in a broad range of taxa.

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Data Accessibility

COI gene sequence and raw ddRAD/two-enzyme GBS sequencing files will be made available on GenBank and NCBI SRA, respectively, pending manuscript acceptance. Data citation: Campbell *et al.* (2020).

Author contributions

EOC and ZGM conducted data analyses and wrote the initial manuscript draft; all authors contributed to study design, specimen collection, and manuscript draft revisions.

Tables and Figures

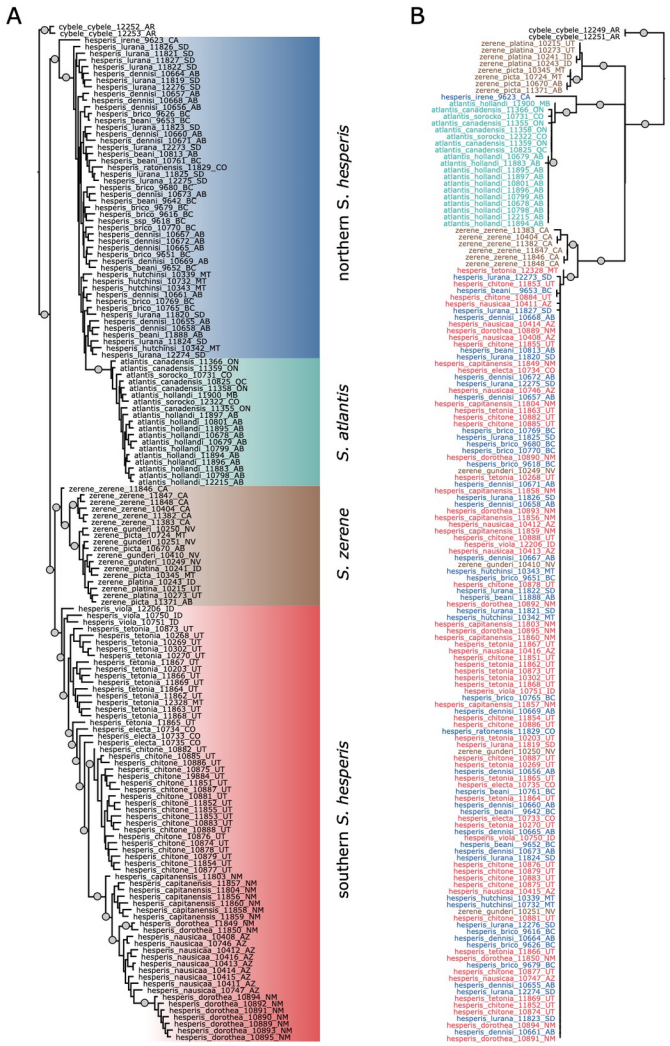

Figure 1: Maximum likelihood phylogenies based on (A) nuclear SNPs and (B) mitochondrial *COI* sequence. Specimens in (B) are coloured according to their group membership in (A). Grey circles on nodes indicate bootstrap values > 75%.

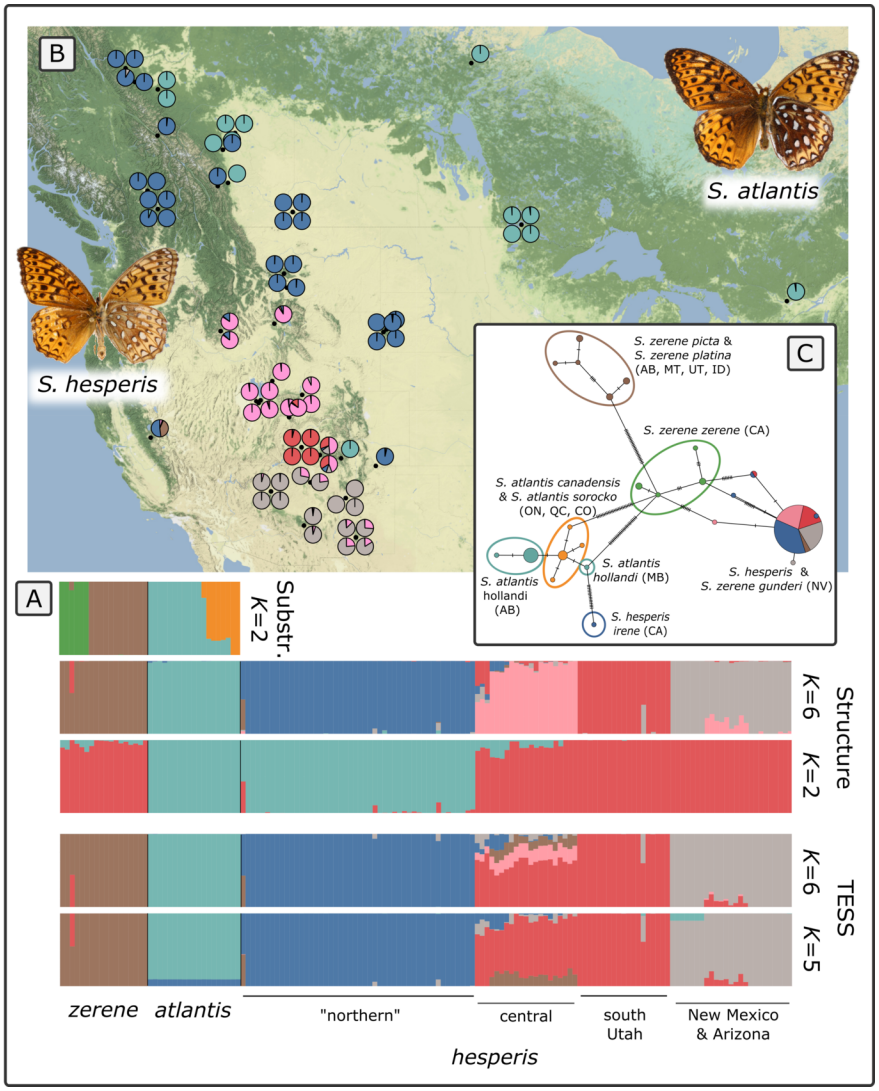

Figure 2: Geographic assessment of population genetic clustering of *S. atlantis* and *S. hesperis* using SNPs. Structure and TESS results (A) consistently indicate a major genetic divergence between northern and southern *S. hesperis* lineages. Populations of *S. hesperis* and *S. atlantis* identified in the K =6 Structure analysis are plotted as pie charts on the map in (B) to show correspondence between genetic and geographic structure. The *COI* haplotype network for the same specimens (C) depicts a lack of geographic structure in *S. hesperis* and haplotype sharing between *S. hesperis* and Nevadan *S. zerene* . Hatches along the branches in (C) indicate the number of nucleotide differences between sequences, and haplotype colours correspond to the Structure clustering and subclustering results in (A). Top right image: *S. atlantis hollandi* ; lower left image: *S. hesperis beani* . The dorsal wing surface is shown on the left side of each specimen, and the ventral surface is depicted on the right.

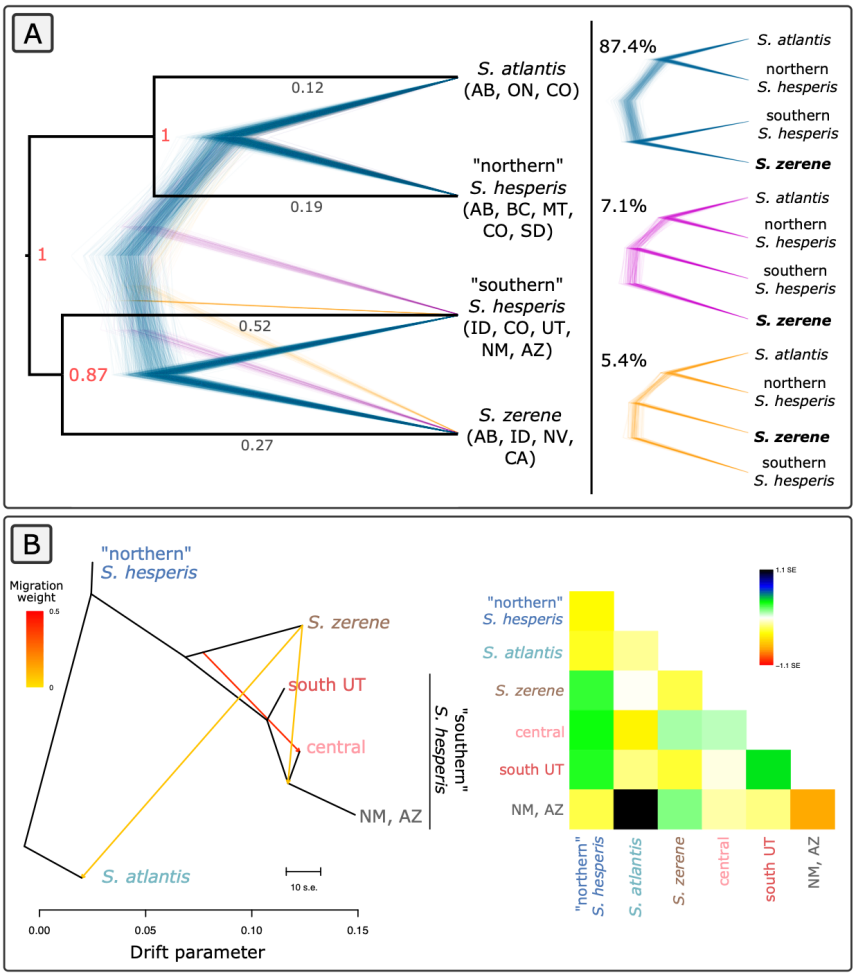

Figure 3: BFD* species delimitation and TreeMix introgression results using SNPs. The left panel in (A) depicts the BFD* "4 species" model visualized as both a maximum clade credibility (MCC) tree (overlaid in black), and as a "tree cloud" output by DensiTree (in colour), supporting northern and southern *S. hesperis* as distinct species. Posterior probability values are indicated in red at both nodes on the MCC tree, and grey values along the branches indicate the estimates of theta ($=N_e$) for each lineage. The right panel in (A) visualizes the genealogical discordance between SNPs as the source of poor branch support for the *S. zerene* /southern*S. hesperis* clade on the MCC tree, and indicates three alternate relationships between *S. zerene* and *S. atlantis/S. hesperis*. Percentages above each DensiTree represent the proportion of trees in the posterior distribution depicting that topology. (B) depicts the maximum likelihood phylogeny (left) output by TreeMix using the populations recovered in the $K = 6$ Structure analysis (Fig. 2), with three statistically significant migration events showing putative introgression between populations of *S. zerene*, *S. hesperis*, and*S. atlantis* . The model residuals for this analysis are indicated by the heatmap on the right in (B).

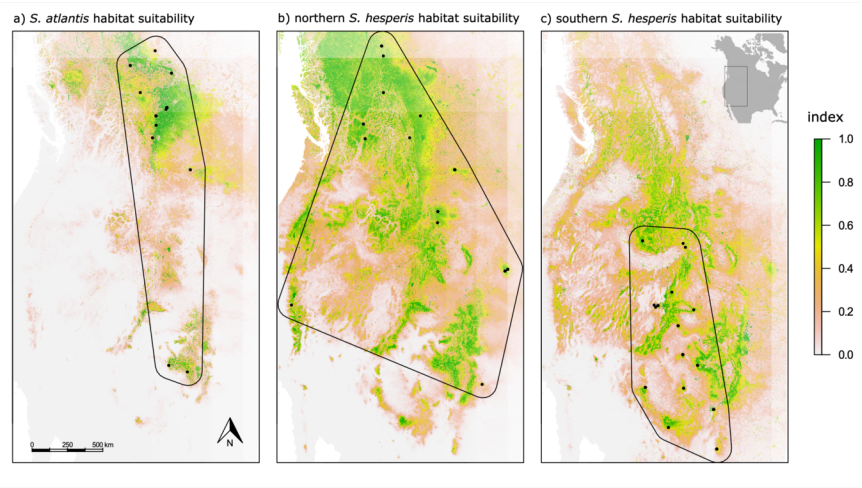

Figure 4: Predicted habitat suitability surfaces based on ENMs for (A) *Speyeria atlantis* , (B) northern *S. hesperis* , and (C) southern *S. hesperis* . For each lineage, five ENMs were built, each withholding a different 20% of occurrence localities used for model evaluation. Background points (n = 10,000) were generated within each lineage's buffered minimum convex polygon to sample available habitat. The average of the five models were used to predict habitat suitability across the entire study area. Each 1-km grid cell received a habitat suitability score ranging from 0–1, with higher values indicating higher suitability.

BFD* model	Species groups tested	MLE	BF
a priori	"*S. hesperis*", "*S. atlantis*", "*S. zerene*"	-13863.6	
"2 species"	"north *S. hesperis* + *S. atlantis*", "south *S. hesperis* + *S. zerene*"	-14397.5	1067.8
"3 species"	"*S. atlantis* + north *S. hesperis*", "south *S. hesperis*", "*S. zerene*"	-13814.8	-97.6
"4 species"	*"S. atlantis"*, *"S. zerene"*, "north *S. hesperis"*, "south *S. hesperis"*	-13462.3	-802.6
"6 species"	"*S. zerene*", *"S. atlantis*", "north *S. hesperis*", "central", "south UT", "NM & AZ"	-22657.1	17587

Table 1: BFD* model selection results. The highest marginal likelihood estimation (MLE) and most negative Bayes Factor (BF) values indicate strongest model support; the "4 species" model was therefore considered optimal.

	S. atlantis	*S. atlantis*	northern *S. hesperis*	northern *S. hesperis*	southern
Predictor variable	mean	s.e.	mean	s.e.	mean
terrain ruggedness	6.58	10.07	15.13	10.5	4.06
heat load	0.84	0.44	0.28	0.62	0.18
land cover	54.78	13.97	35.04	12.79	39.52
mean temperature of the coldest month	0	0	0	0	0
mean temperature of the warmest month	9.99	15.87	3.1	5.1	0
continentality	0	0	0.56	1.26	0
chilling degree days	1.89	2.61	0	0	4.46
growing degree days	19.54	18.74	42.71	20.12	41.59
extreme minimum temperature	4.73	5.44	0	0	0
precipitation as snow	0.19	0.43	0.17	0.38	3.97
summer (Jun to Aug) precipitation	1.47	1.9	3.02	3.98	6.22

Table 2: The relative contribution (%) of geographic and environmental predictor variables to *Speyeria atlantis* , northern*S. hesperis* , and southern *S. hesperis* ecological niche models (ENMs). Geographic predictor variables included terrain ruggedness, heat load (based on terrain slope and aspect), and land cover (12 categories). Environmental predictor variables included mean temperature of the coldest month, mean temperature of the warmest month, the difference between mean temperatures of the coldest and warmest months (continentality), degree-days below 0°C (chilling degree days), degree-days above 5°C (growing degree days), extreme minimum temperature, precipitation as snow, and summer (Jun to Aug) precipitation. Relative contribution was measured as the drop in AUC values after each variable was randomly permuted. For each of the three lineages, five ENMs were built, each withholding a different 20% of occurrence localities used for model evaluation. The mean and standard deviation (s.e.) of relative contributions for each predictor variable across the five ENMs are reported.