BIOLOGY LETTERS

royalsocietypublishing.org/journal/rsbl

Research

Cite this article: Nice CC, Fordyce JA, Bell KL, Forister ML, Gompert Z, DeVries PJ. 2019 Vertical differentiation in tropical forest butterflies: a novel mechanism generating insect diversity? *Biol. Lett.* **15**: 20180723. http://dx.doi.org/10.1098/rsbl.2018.0723

Received: 14 October 2018
Accepted: 8 December 2018

Subject Areas:
ecology, evolution

Keywords:
genomic differentiation, vertical stratification, butterfly diversity, tropical forests

Author for correspondence:
Chris C. Nice
e-mail: ccnice@txstate.edu

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4341143.

THE ROYAL SOCIETY PUBLISHING

Evolutionary biology

Vertical differentiation in tropical forest butterflies: a novel mechanism generating insect diversity?

Chris C. Nice[1], James A. Fordyce[2], Katherine L. Bell[1], Matthew L. Forister[3], Zachariah Gompert[4] and Phil J. DeVries[5]

[1]Department of Biology, Population and Conservation Biology Program, Texas State University, San Marcos, TX 78666, USA
[2]Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996, USA
[3]Department of Biology, Program in Ecology, Evolution and Conservation Biology, University of Nevada, Reno, NV 89557, USA
[4]Department of Biology, Utah State University, Logan, UT 84322, USA
[5]Department of Biology, University of New Orleans, New Orleans, LA 70148, USA

CCN, 0000-0001-9930-6891; MLF, 0000-0003-2765-4779

Many tropical fruit-feeding nymphalid butterflies are associated with either the forest canopy or the understorey; however, the exceptions offer insights into the origins of tropical diversity. As it occurs in both habitats of tropical forests in Ecuador and Peru, *Archaeoprepona demophon* is one such exception. We compared patterns of occurrence of *A. demophon* in the canopy and understorey and population genomic variation for evidence of ecological and genetic differentiation between habitats. We found that butterfly occurrences in the canopy were largely uncorrelated with occurrences in the understorey at both localities, indicating independent demographic patterns in the two habitats. We also documented modest, significant genome-level differentiation at both localities. Genetic differentiation between habitat types (separated by approx. 20 m in elevation) was comparable to levels of differentiation between sampling locations (approx. 1500 km). We conclude that canopy and understorey populations of *A. demophon* represent incipient independent evolutionary units. These findings support the hypothesis that divergence between canopy and understorey-associated populations might be a mechanism generating insect diversity in the tropics.

1. Introduction

The mechanisms generating high species diversity in tropical systems are poorly understood [1–3]. One emerging feature of tropical forest systems is the vertical stratification of their biota [4], which is well documented in butterfly communities of lowland tropical forests [5–7]. Trap studies of fruit-feeding nymphalid butterflies (Nymphalidae), which primarily use rotting fruit for adult nutrition, have demonstrated that most species are strongly associated with either the forest canopy or the understorey [1]. This apparent specialization is correlated with a strong phylogenetic signal, such that canopy species are generally more closely related to other canopy species than to understorey species, and vice versa [5]. This supports the hypothesis that adaptation to canopy or understorey habitat has strongly influenced the patterns of evolutionary divergence among neotropical forest butterflies. However, some species are exceptions and have been observed with equal frequency in both habitats [1,5]. These species might be habitat generalists, or, they might represent cases of incipient differentiation, with divergence being propelled by local adaptation to the unique environmental conditions in each habitat.

© 2019 The Author(s) Published by the Royal Society. All rights reserved.

Table 1. Sample collection information for 82 *Archaeoprepona demophon* that were examined for population genetic variation. *N*: total sample size; female: number of females; male: number of males.

locality	collection date	*N*	female	male
Garza Cocha, La Selva Lodge, Ecuador				
canopy	October 1999–July 2001	20	6	14
understorey	October 1999–July 2002	22	12	10
Los Amigos Biological Station, Peru				
canopy	April 2004–August 2005	10	4	6
understorey	April–November 2004	30	6	24

Specialization and the consequent reduction in gene flow would, at least partially, uncouple canopy and understorey populations demographically and facilitate genetic differentiation. Here we examine datasets from tropical fruit-feeding butterfly communities to assess patterns of occurrence in canopy versus understorey and identify a focal species that occupies both habitats. We combine occurrence data with genome-level population genetic data to ask whether there is evidence of ecological and genetic differentiation between canopy and understorey populations of our focal species. Specifically, we quantified phenological and demographic independence, and the patterns of genomic differentiation, between canopy and understorey populations.

2. Material and methods

(a) Butterfly biology and demography

Fruit-feeding nymphalid butterflies were sampled using paired, baited traps, placed in the canopy (greater than 20 m elevation) and understorey (1 m above the forest floor), sampled for 5 consecutive days during the first 10 days of each month (see [1] for details) at Tirimbina Biological Reserve, Costa Rica (10.415°, −84.120°), Garza Cocha, La Selva Lodge, Ecuador (−0.498°, −76.373°), Shiripuno Research Center, Ecuador (−1.105°, −76.731°) and Los Amigos Biological Station, Peru (−12.609°, −70.092°) [1,5]. Species identifications were made by P.J.D. and Isidro Chacon, and specimens are deposited at the University of New Orleans, USA or Museo Nacional, San Jose, Costa Rica. To describe vertical habitat separation of communities and to identify candidate focal species, we assessed the tendency of butterfly species to occur in either the canopy or understorey using these long-term trap data. Habitat fidelity for each of 139 species was characterized using a hierarchical Bayesian model on monthly count data, where capture in the canopy or understorey was modelled as a binomial distribution. Analyses were conducted using the STAN language [8] (see electronic supplementary material for STAN code).

Based on our estimates of habitat fidelity (see Results), we chose *Archaeoprepona demophon* as our focal species. We examined demographic connectivity between *A. demophon* in the canopy and understorey using the partial correlation between abundances in each habitat across months at each site after accounting for total butterfly abundance, which can vary across months [9].

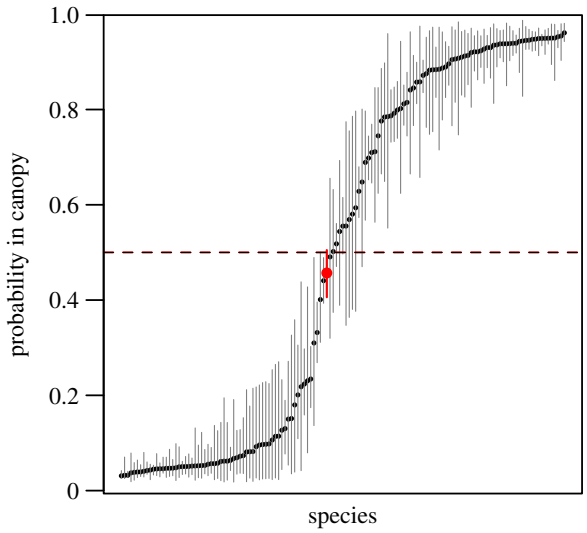

Figure 1. Estimates of the probability of occurrence in the canopy for 139 fruit-feeding nymphalid butterflies. Dots represent point estimates (medians) and bars represent 95% highest-density intervals for probability of occurrence in the canopy. The focal species, *Archaeoprepona demophon*, is indicated in red. The dashed line indicates equal probability of occurring in the canopy and understorey.

(b) Molecular methods

We used a genotyping-by-sequencing protocol to generate markers throughout the genome of *A. demophon*. DNA was extracted from a single leg from each of 82 individuals from Garza Cocha, La Selva Lodge, Ecuador and Los Amigos Biological Station, Peru (table 1), and a reduced representation genomic library was produced for each individual following the methods of Gompert *et al*. [10] and Parchman *et al*. [11] (see electronic supplementary material for details). Briefly, genomic DNA was digested with two restriction enzymes, and multiplex identifiers and adapters were ligated to the resulting fragments, which were then amplified with PCR using the Illumina primers. PCR products were then pooled, and fragments of between 300 and 400 bp were selected with a Blue Pippin (Sage Science) and sequenced on the Illumina 2500 platform using single-end reads of 100 bp in length. A de novo assembly was performed as described in the *dDocent* variant-calling pipeline [12] and the scaffolds from this de novo assembly were treated as artificial chromosomes for a reference-based assembly in which all sequence reads were assembled using bwa 0.7.5a-r405 [13]. SAMtools v. 0.1.19 [14] was used to index, sort and merge the individual alignments. Bi-allelic, single nucleotide sites were identified and filtered using SAMtools.

We measured genetic differentiation between canopy and understorey samples and between localities (Ecuador and Peru) using two approaches. First, we calculated pairwise Nei's G_{ST}, a multi-locus version of Wright's F_{ST} [15], which we hereafter call F_{ST}. In a second approach, we used a distance-based redundancy analysis (dbRDA) [16] on the genotypic dissimilarity calculated as pairwise Euclidean distances among individuals based on their multi-locus genotypes using the vegan package [17]. Statistical significance of the model was assessed via 99 999 permutations of the data. All calculations were performed in R v. 3.4.3 [18].

3. Results

(a) Patterns of occurrence

Estimates of habitat fidelity indicated substantial variation among species, where the majority showed a strong preference

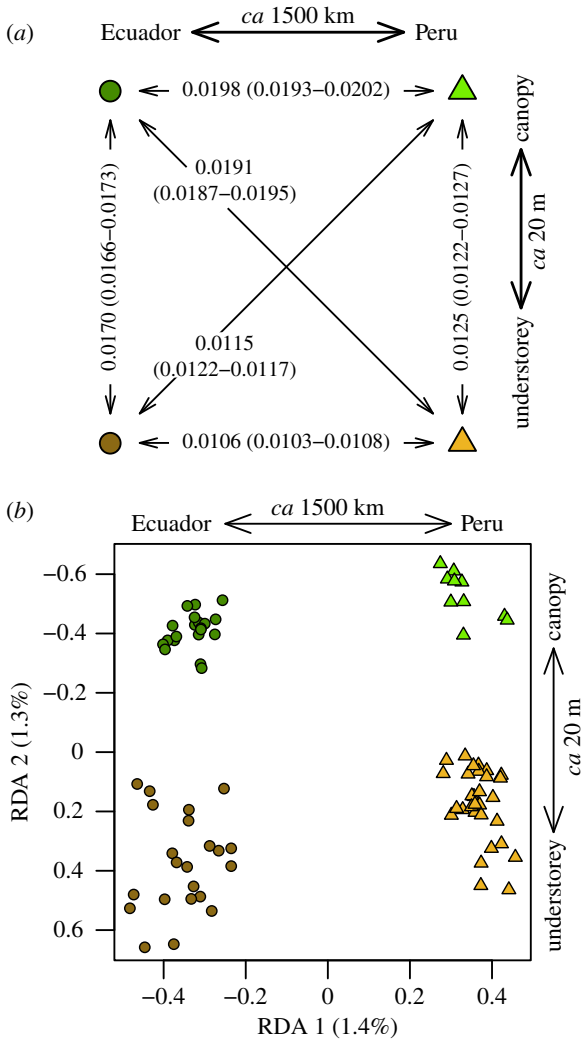

Figure 2. Patterns of genomic differentiation among localities for *Archaeoprepona demophon* genotyped for 43 799 SNP loci. (*a*) Estimates of pairwise F_{ST} (and bootstrap confidence intervals) are provided for each comparison. Differentiation between canopy and understorey samples (separated by approx. 20 m) is comparable to differentiation between Ecuador and Peru (approx. 1500 km). (*b*) Redundancy analysis (RDA) of 82 sampled individuals of *A. demophon* separates canopy and understorey samples as well as Ecuador and Peru. Symbols represent individuals whose positions in canonical ordination space are determined by their multi-locus genotypes. Circles indicate individuals from Ecuador, triangles indicate individuals from Peru, green symbols represent individual samples from the canopy, brown from the understorey.

for either canopy or understorey (figure 1; electronic supplementary material, table S1; see also [5] for a detailed examination of these patterns). We chose *A. demophon* as our focal species because it occurred in both habitats and had an intermediate estimated habitat fidelity (posterior probability of being found in canopy (median (95% highest-density interval (HDI)) = 0.46 (0.41, 0.50)) and based on the availability of specimens. We did not detect a correlation between canopy and understorey abundances of *A. demophon* across sites (correlation coefficients r (95% confidence intervals), t-statistics and p-values: Costa Rica: $r = 0.11$ $(-0.09,\ 0.31)$, $t_{96} = 1.1251$, $p = 0.2634$; Peru: $r = 0.26$ $(-0.17,\ 0.61)$, $t_{21} = 1.2326$, $p = 0.2314$; Ecuador (Shiripuno): $r = -0.39$ $(-0.74,\ 0.13)$, $t_{14} = -1.5816$, $p = 0.1361$; Ecuador (Garza Cocha): $r = 0.17$ $(-0.01,\ 0.35)$, $t_{110} = 1.8577$, $p = 0.06589$), evidence that the canopy and understorey are not exhibiting demographic patterns expected of a single population.

Table 2. Results of RDA significance tests using a permutational ANOVA. d.f.: degrees of freedom, variance: variance partitioning between 'country' (Ecuador versus Peru) and 'forest' (canopy versus understorey), *F*: pseudo *F* from 99 999 permutations of individuals among localities, *p*-value: probability that the pseudo *F* is at least as large under the null hypothesis of no association between factors (country or forest) and individual genotypes.

factor	d.f.	variance	*F*	*p*-value
country	1	59.3	1.0872	0.005
forest	1	55.9	1.0242	0.083
residual	79	4311.5		

(b) Genomic differentiation

Following reference-based assembly, variant-calling and filtering, we analysed a final dataset of 43 799 single-nucleotide polymorphism (SNP) loci. The median sequence depth was 26.42 (s.e. = 0.28) reads per individual per locus. All pairwise F_{ST} values were small, ranging from 0.0106 to 0.0198, but significantly different from zero (figure 2*a*). The ordination using RDA illustrated patterns similar to the pairwise F_{ST} (figure 2*b*, table 2). The overall model was significant ($p < 0.001$), 1.4% of the variation explained between Ecuador and Peru ($p = 0.005$) and 1.3% of the variation explained by canopy versus understorey ($p = 0.083$), which is not significant ($\alpha = 0.05$). The sample imbalance in Peru ($n_{\text{canopy}} = 10$, $n_{\text{understorey}} = 30$) likely limits our power to reject the null hypothesis for canopy versus understorey in this model (see electronic supplementary material for the exploration of power). Separate analyses for each location showed significant differentiation between canopy and understorey in Ecuador ($p = 0.037$) but failed to detect a difference in Peru ($p > 0.1$).

4. Discussion

Individuals of the butterfly *A. demophon* captured in traps from Costa Rica, Peru and two sites in Ecuador were nearly equally likely to be sampled in the canopy and understorey (the probability of being found in the canopy was estimated to be 0.46 (95% HDI: 0.41, 0.50)). Thus, *A. demophon* is among a few fruit-feeding nymphalid butterflies in neotropical forests that are not exclusively associated with the canopy or the understorey. Furthermore, *A. demophon* canopy populations appear to be demographically uncorrelated and unconnected with understorey populations. Examination of patterns of population genomic variation in canopy and understorey samples from Ecuador and Peru shows low, but measurable, differentiation (figure 2). Differentiation between canopy and understorey samples (separated by approx. 20 m) is comparable to the differentiation between Ecuador and Peru (approx. 1500 km) (figure 2). Together, these results support the hypothesis that local adaptation to canopy and understorey habitats might be a mechanism of population differentiation in tropical forests.

While we do not understand the mechanism(s) that might drive local adaptation in canopy and understorey habitats, the pattern of genetic variation we observed for *A. demophon* is consistent with the concept of isolation by environment [19], wherein differences among habitats can have a role in determining population genetic structure that is similar to

isolation by distance [20]. There is at least one example of vertical partitioning between sibling species that might have arisen from similar population processes. The fruit-feeding nymphalids *Colobura dirce* and *C. annulata* were considered members of the same species until an extensive examination of their morphology and natural history [21]. Despite minor morphological divergence in adults, larvae of these species differ in colour, and understorey *C. dirce* lays solitary eggs on saplings, while canopy *C. annulata* lays large clutches in mature trees of the same host plant. Examination of *A. demophon* revealed no morphological differences between canopy and understorey adults of either sex, or between samples from Ecuador and Peru (C. Penz, University of New Orleans, personal communication).

Much remains to be learned in this system. In particular, we do not know if the amount of observed differentiation is the result of habitat-specific selection acting on demographically linked subpopulations in equilibrium, or if the differentiation marks the start of a process of speciation that would ultimately result in isolated species in the canopy and understorey. Nevertheless, the patterns of demographic and genomic differentiation within this nominal species parallel phylogenetic patterns at the community level [5] and support the hypothesis that adaptation to vertically distributed habitats in tropical forests might contribute to the diversity of these communities. Comparable investigations of other taxa (e.g. other butterflies as well as other tropical forest organisms in general) are required to assess the generality of this pattern.

Ethics. This study was carried out in compliance with relevant guidelines related to research on insects.

Data accessibility. Data are available in the electronic supporting information and on Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad.rq0pj53 [22].

Authors' contributions. P.J.D., J.A.F., Z.G. and C.C.N. designed the study; P.J.D., K.L.B. and C.C.N. generated the data; J.A.F., K.L.B., M.L.F., Z.G. and C.C.N. analysed the data; all authors contributed to interpretation of results and writing and editing the manuscript. In addition, all authors have given their approval for the publication of this manuscript and have agreed to be accountable for the accuracy and integrity of the work.

Competing interests. The authors have no competing interests.

Funding. This research was funded by the National Science Foundation (DEB-1638768 to Z.G., DEB-1638773 to C.C.N., DEB-1638922 to J.A.F. and DEB-1638793 to M.L.F.) and by the Office of the Associate Vice President for Research, Texas State University.

Acknowledgements. We thank Isidro Chacon, Chevo Cascante, Cristian Miranda, Sergio Padilla and Emmanuel Rojas for assistance in Costa Rica; Caroline Dingle, Casey Dunn, Chris Funk, Harold Greeney, Rodney Guerra, Ryan Hill, Guillermo Hualinga, Jose Hualinga, Trond Larsen, Carlos Licuey and Thomas Walla for assistance in Ecuador and Peru; and Alex Buerkle, Lauren Lucas, Ben Fitzpatrick, Todd Pierson and the EEB group at Texas State University for comments and discussion. We especially thank C. Penz for morphological examination of specimens used for genetic analyses and for comments on an earlier version of this manuscript. We thank Texas State University for the use of the LEAP computing cluster.

References

1. DeVries PJ, Walla TR. 2001 Species diversity and community structure in neotropical fruit-feeding butterflies. *Biol. J. Linn. Soc.* **74**, 1–15. (doi:10.1111/j.1095-8312.2001.tb01372.x)
2. Mittelbach GG *et al.* 2007 Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography. *Ecol. Lett.* **10**, 315–331. (doi:10.1111/j.1461-0248.2007.01020.x)
3. Forister ML *et al.* 2015 The global distribution of diet breadth in insect herbivores. *Proc. Natl Acad. Sci. USA* **112**, 442–447. (doi:10.1073/pnas.1423042112)
4. Basset Y *et al.* 2015 Arthropod distribution in a tropical rainforest: tackling a four dimensional puzzle. *PLoS ONE* **10**, e0144110. (doi:10.1371/journal.pone.0144110)
5. Fordyce JA, DeVries PJ. 2016 A tale of two communities: neotropical butterfly assemblages show higher beta diversity in the canopy compared to the understory. *Oecologia* **181**, 235–243. (doi:10.1007/s00442-016-3562-0)
6. Fermon H, Waltert M, Mühlenberg M. 2003 Movement and vertical stratification of fruit-feeding butterflies in a managed West African rainforest. *J. Insect Conserv.* **7**, 7–19. (doi:10.1023/A:1024755819790)
7. Molleman F, Kop A, Brakefield PM, Zwaan BJ. 2006 Vertical and temporal patterns of biodiversity of fruit-feeding butterflies in a tropical forest in Uganda. *Biodiversity Conserv.* **15**, 107–121. (doi:10.1007/s10531-004-3955-y)
8. Carpenter B *et al.* 2017 Stan: a probabilistic programming language. *J. Stat. Softw.* **76**, 1–32. (doi:10.18637/jss.v076.i01)
9. Grøtan V, Lande R, Engen S, Sæther BE, DeVries PJ. 2012 Seasonal cycles of species diversity and similarity in a tropical butterfly community. *J. Anim. Ecol.* **81**, 714–723. (doi:10.1111/j.1365-2656.2011.01950.x)
10. Gompert Z, Lucas LK, Nice CC, Fordyce JA, Forister ML, Buerkle CA. 2012 Genomic regions with a history of divergent selection affect fitness of hybrids between two butterfly species. *Evolution* **66**, 2167–2181. (doi:10.1111/j.1558-5646.2012.01587.x)
11. Parchman TL, Gompert Z, Mudge J, Shilkey FD, Benkman CW, Burkle CA. 2012 Genome-wide association genetics of an adaptive trait in lodgepole pine. *Mol. Ecol.* **21**, 2991–3005. (doi:10.1111/j.1365-294X.2012.05513.x)
12. Puritz JB, Hollenbeck CM, Gold JR. 2014 *dDocent*: a RADseq, variant-calling pipeline designed for population genomics of non-model organisms. *PeerJ* **2**, e431. (doi:10.7717/peerj.431)
13. Li H, Durbin R. 2009 Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**, 1754–1760. (doi:10.1093/bioinformatics/btp324)
14. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009 The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078–2079. (doi:10.1093/bioinformatics/btp352)
15. Nei M. 1987 *Molecular evolutionary genetics*. New York, NY: Columbia University Press.
16. Legendre P, Legendre L. 2012 *Numerical ecology*, 3rd edn. Amsterdam, The Netherlands: Elsevier Sciences.
17. Oksanen J *et al*. 2013 *vegan: community ecology package*, version 2.0-10. See http://CRANR-projectorg/package=vegan.
18. R Development Core Team. 2012 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See https://www.r-project.org.
19. Wang IJ, Bradburd GS. 2014 Isolation by environment. *Mol. Ecol.* **23**, 5649–5662. (doi:10.1111/mec.12938)
20. Wright S. 1943 Isolation by distance. *Genetics* **28**, 114–138.
21. Willmott K, Constantino L, Hall JPW. 2001 A review of *Colobura* (Lepidoptera: Nymphalidae) with comments on larval and adult ecology and description of a sibling species. *Ann. Entomol. Soc. Am.* **94**, 185–196. (doi:10.1603/0013-8746(2001)094[0185:AROCLN]2.0.CO;2)
22. Nice CC, Fordyce JA, Bell KL, Forister ML, Gompert Z, DeVries PJ. 2018 Data from: Vertical differentiation in tropical forest butterflies: a novel mechanism generating insect diversity? Dryad Digital Repository. (doi:10.5061/dryad.rq0pj53)