

Genetic differentiation associated with host plants and geography among six widespread species of South American *Blepharoneura* fruit flies (Tephritidae)

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Abstract

Tropical herbivorous insects are astonishingly diverse, and many are highly host-specific. Much evidence suggests that herbivorous insect diversity is a function of host plant diversity; yet, the diversity of some lineages exceeds the diversity of plants. Although most species of herbivorous fruit flies in the Neotropical genus *Blepharoneura* are strongly host-specific (they deposit their eggs in a single host plant species and flower sex), some species are collected from multiple hosts or flowers and these may represent examples of lineages that are diversifying via changes in host use. Here, we investigate patterns of diversification within six geographically widespread *Blepharoneura* species that have been collected and reared from at least two host plant species or host plant parts. We use microsatellites to (1) test for evidence of local genetic differentiation associated with different sympatric hosts (different plant species or flower sexes) and (2) examine geographic patterns of genetic differentiation across multiple South American collection sites. In four of the six fly species, we find evidence of local genetic differences between flies collected from different hosts. All six species show evidence of geographic structure, with consistent differences between flies collected in the Guiana Shield and flies collected in Amazonia. Continent-wide analyses reveal – in all but one instance – that genetically differentiated flies collected in sympatry from different host species or different sex flowers are not one another's closest relatives, indicating that genetic differences often arise in allopatry before, or at least coincident with, the evolution of novel host use.

Introduction

Understanding how biodiversity is generated is a major goal of evolutionary biology. Insects are the most diverse group of multicellular organisms on Earth (Grimaldi & Engel, 2005), and their diversity peaks in the tropics (Price, 2002; Hillebrand, 2004); yet, most research on insect speciation has focused on insects in

temperate zones (May, 1990). Estimates of global insect species richness span the wide range of 6 million to 30 million (Erwin, 1982; Hamilton *et al.*, 2010, 2011), and the diversity in tropical forests is even less certain (Novotny & Miller, 2014). Such high diversity may be explained, in part, by ecological specialization (Novotny *et al.*, 2006; although see Dyer *et al.*, 2007), which can reduce niche overlap between sympatric populations (Bush, 1993; Nosil, 2012); however, specialization and the minimization of niche overlap may not fully explain high levels of Neotropical insect diversity. In a group of highly diverse host-specific tephritid fruit flies (*Blepharoneura* Loew), we find both extreme

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specialization and extreme niche overlap (Condon *et al.*, 2014). In this group of insects, divergence in allopatry (often without changes in host use) may help explain patterns of diversity (Condon *et al.*, 2008b).

If a group of herbivorous insects includes many specialists that feed on different hosts or different host tissues, the evolutionary history of that group of insects likely involves shifts in host use. Most evidence suggests that patterns of host use do not reflect cospeciation of host plants and insects (although see Cruaud *et al.*, 2012); instead, insects often appear to diverge coincident with their shifts to novel hosts (*e.g.* Winkler *et al.*, 2009; Wilson *et al.*, 2012; de Vienne *et al.*, 2013). This trend is not as clear for *Blepharoneura* flies, whose larvae tend to be specialized feeders on specific plant tissues. Phylogenetic analyses of more than 40 *Blepharoneura* species show that shifts to new host plant species or to differentiated tissues of male vs. female flowers on the same plant species – host plants in the family Guraniinae are highly sexually dimorphic, with morphologically distinct male and female flowers (Condon & Gilbert, 1988) – sometimes coincide with diversification, but divergence without host shifts is also common, and many different sympatric *Blepharoneura* species often overlap on the same host (Fig. 1; Condon *et al.*, 2008b; Condon *et al.*, 2014). What, then, is the relationship between diversity, allopatric divergence, and host shifts for *Blepharoneura* flies?

Previous genetic analyses of *Blepharoneura* diversification patterns used sequence data from mitochondria and conserved nuclear genes (Condon *et al.*, 2008b). Although those data yielded fairly robust phylogenies, they may not be useful for revealing patterns of current and recent gene flow: incomplete lineage sorting or introgression can confound signals at tips of trees (Funk & Omland, 2003). Further, within-species genetic structure associated with use of different plant tissues (a signature of a potential host shift) may not be detectable with simple sequence data. The goal of the present study was to use a more sensitive marker system (microsatellites) to evaluate patterns of recent and ongoing genetic differentiation below the species level for six geographically widespread *Blepharoneura* species that include specimens collected from more than one habitat niche (*i.e.* different plant species and/or different sex flowers of the same plant species). We were particularly interested in the genetic structure of flies of each species that had been reared from multiple different plant species or flower types because these may (or may not) be individuals belonging to populations in the early stages of a host shift.

For each of the six focal species, we scored a suite of microsatellite loci and then used Bayesian clustering algorithms to evaluate ecological and geographic correlates of genetic structure both at local geographic sites and across each species' South American range. Our goals were to assess (1) patterns of gene flow among

sympatric flies collected from different host plant species or plant tissues and (2) patterns of genetic structure across tropical South America.

Materials and methods

Organisms and samples

Blepharoneura is a diverse genus (>100 species) of tephritid fruit flies with endophagous larvae that feed inside tissues of plants in the Cucurbitaceae (Condon & Norrbom, 1999; Condon *et al.*, 2008b; Norrbom and Condon 2010). One especially diverse clade within *Blepharoneura* includes specialists that deposit eggs within the calyx tissue of flowers of species in a Neotropical subtribe of functionally dioecious species (Guraniinae) (Condon *et al.*, 2008b). Larvae feed on tissue of either male or female flowers. Using data from a previous study (Condon *et al.*, 2008b), we chose six *Blepharoneura* 'species' (monophyletic groups having maximum pairwise differences $\leq 4\%$ mtCOI divergence; Condon *et al.*, 2008b), each of which included collections that revealed some variation in patterns of host use. We chose species that included individuals collected as larvae hidden in calyx tissue of flowers from more than one plant species or both flower sexes from diverse geographic locations. These six species are here referred to as *Blepharoneura* sp1, *B.* sp4, *B.* sp8, *B.* sp10, *B.* sp21 and *B.* sp30, as in Condon *et al.* (2008b). Flies were assigned to species based on characteristic mtCOI haplotypes as in Condon *et al.* (2008b, 2014). The six species are not sister species, but all are part of a large clade (30 species) that specializes primarily on flower tissue of species in the Guraniinae (Fig. 1; Condon *et al.*, 2008b). Because we were interested in assessing genetic structure associated with geography and host, we chose specimens collected from diverse countries (all countries where members of a given species had been sampled), and from each species, we chose a subset of individuals reared from different host plant species or host-sex flower. To test for structure associated with plant hosts (or flower sex), we intentionally included specimens from 'rarely used hosts'; thus, the proportion of flies in our sample associated with each host plant species (or flower sex) does not always reflect the proportions found in nature (Table 1).

Samples represent flies from 16 collection sites across seven countries in South America: Bolivia, Brazil, Ecuador, French Guiana, Peru, Suriname and Venezuela. Collection sites are defined here as collections made from plants found within 12 km of one another, but often individual vines of different species can be found climbing on top of each other. All but four individual flies (two *B.* sp.8 individuals collected from *Gurania eriantha* (Poepp. & Endl.) Cogn. and two *B.* sp.10 collected from *Gurania robusta* Suesseng.) were collected from male or female flowers of *Gurania acuminata* Cogn. or

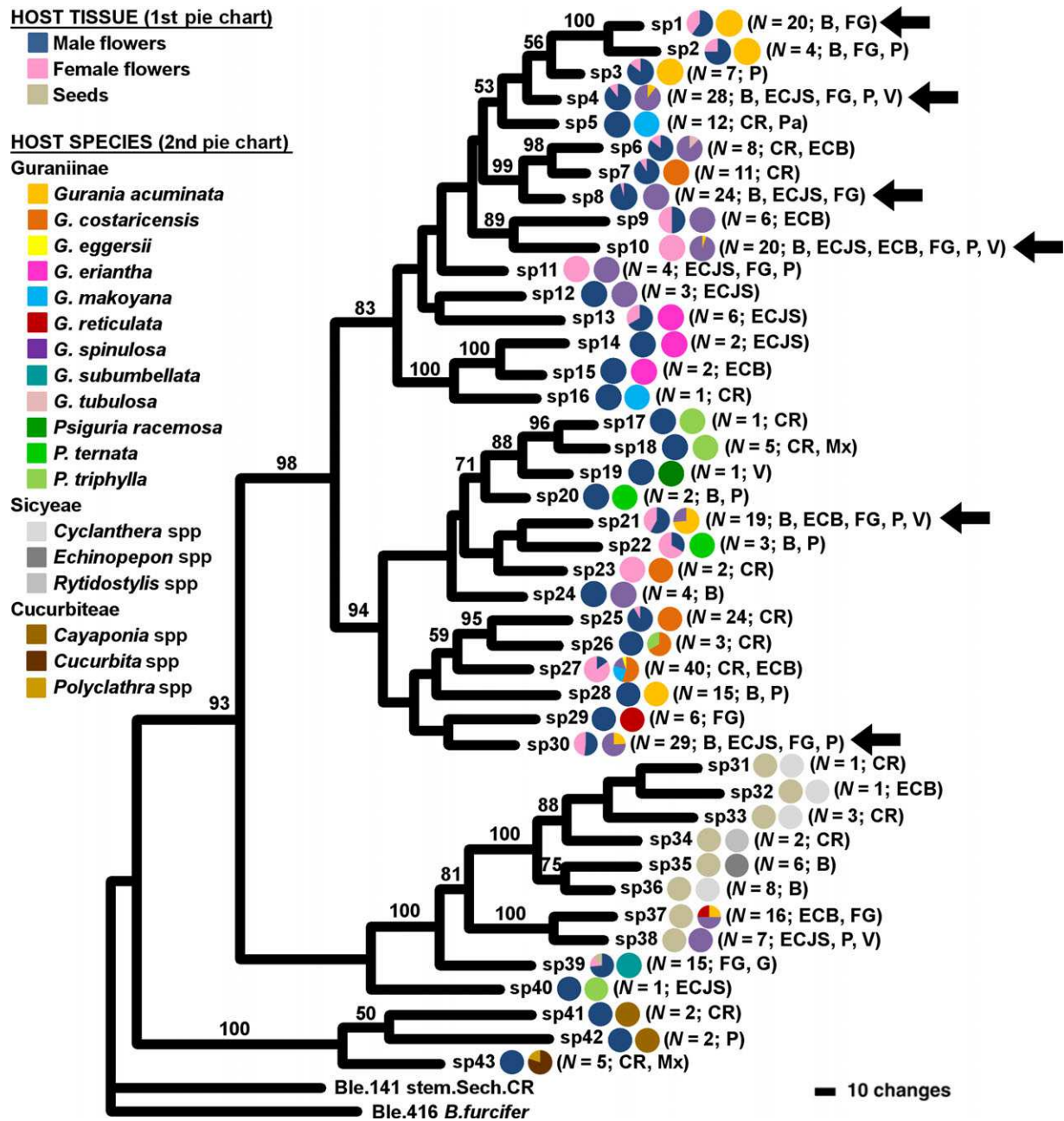


Fig. 1 ML tree (adapted from Condon *et al.*, 2008b) of 43 species of *Blepharoneura* shows that the six species included in microsatellite analyses are not sister species. This tree is based on ML analysis of mitochondrial sequences (mtCOI) and two nuclear loci (EF-1 α and CAD) from geographically widespread collections of reared flies; pie charts at branch tips illustrate the proportions of flies in each species that were collected from particular host tissues and host plant species as reported in Condon *et al.* (2008b). Bootstrap values (>50%) are included above branches. Bold type and arrows indicate the six species chosen for this study. Geographic distributions are indicated by abbreviations: B = Bolivia; CR = Costa Rica; ECB = Ecuador (west of Andes); ECJS = Ecuador (east of Andes); FG = French Guiana; G = Guyana; Mx = Mexico; P = Peru; V = Venezuela. Fly species chosen for this study (black arrows) were among those that had been collected from two or more different combinations of plant species and tissues and from geographically widespread sites, such that it was possible to assess genetic structure associated with both host use and geographic distance. *Blepharoneura* sp4, sp8 and sp10 are each primarily collected (>80% of all flies) from a single sex flower of a single host species. *Blepharoneura* sp1 was reared from both male and female flowers, but almost always from a single plant species. In contrast, *B. sp21* and sp30 are commonly reared from multiple diverse hosts. More detailed trees (e.g. NJ trees used to delineate conservatively – but arbitrarily – defined species) with information about relationships between flies, host plants and their respective geographic distributions may be found in Condon *et al.* (2008b).

Table 1 Overall patterns of host use by flies ($N = 1110$) representing six species of *Blepharoneura* (Condon *et al.*, 2008b). Samples represent all specimens identified by analysis of mtCOI sequences published here or elsewhere (Condon *et al.*, 2008a,b, 2014). Abbreviations identify host species and host-sex flower from which fly larvae were collected and reared: f = female flower; m = male flower; GA = *Gurania acuminata*; GS = *G. spinulosa*; GE = *G. eriantha*; GI = *G. insolita*; Grob = *G. robusta*.

<i>Blepharoneura</i> species	Sex flower and species of plant host					Total
	mGS	fGS	mGA	fGA	Other	
sp1	2	1	114	21	0	138
sp4	254	12	6	0	0	272
sp8	159	4	3	0	2 (mGE)	168
sp10	6	118	1	1	2 (fGrob)	128
sp21	82	1	50	22	0	155
sp30	134	70	38	6	1	249

Gurania spinulosa (Poepp. & Endl.) Cogn (= *G. lobata* L.), the two most abundant *Blepharoneura* host plants, both of which have widespread geographic distributions. Table S1 shows collection information for samples used in the study.

We used DNA from a total of 692 *Blepharoneura* individuals: *Blepharoneura* sp1 ($N = 104$ individuals); *B. sp4* ($N = 134$); *B. sp8* ($N = 83$); *B. sp10* ($N = 70$); *B. sp21* ($N = 120$); and *B. sp30* ($N = 181$). Each specimen was collected as a larva hidden within the calyx tissue of single host plant flower; larvae are discovered when they emerge from flowers and pupate. Specimens included the following: adult flies that had emerged from puparia and were subsequently preserved in 95% ethanol and stored at -80°C ; EtOH-preserved *Blepharoneura* puparia; or empty *Blepharoneura* puparia from which parasitic wasps had emerged (full description of methods in Condon *et al.*, 2014).

Genomic DNA had previously been extracted and mtCOI had previously been sequenced for 236 flies in our sample (Condon *et al.*, 2008a,b, 2014). For the remaining 456 flies (GenBank Accession #: KX902513-KX902969), we extracted genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). We amplified a 504-bp segment of mtCOI using the primers described in Condon *et al.* (2008a) on a Mastercycler Gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY) with the following programme: 2 min at 92°C , 12 'touchdown' cycles from 58 to 46°C (10 s at 92°C , 10 s at $58-46^{\circ}\text{C}$, 1.5 min at 72°C), 27 cycles at 10 s at 92°C , 10 s at 45°C , 1.5 min at 7°C and 10 min at 72°C . We sequenced segments using Big Dye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA Analyzer (Applied Biosystems) and analysed and aligned sequences using Geneious v.8.17 (Biomatters, Auckland, New Zealand). We generated representative neighbor-joining trees for

each species in Geneious using a Tamura–Nei model. Trees were rooted with two outgroup flies from other *Blepharoneura* species, and bootstraps for each tree were generated using 100 pseudoreplicated data sets. For just one individual (KOF157, a *B. sp21* fly from French Guiana), sequence quality was too poor to include it in a tree.

Microsatellite library construction and scoring

We used the most abundant species in our sample, *B. sp30*, to construct microsatellite libraries. A pooled sample of DNA from 15 adult *B. sp30* flies was sent to the Savannah River Ecology Laboratory (SREL; Aiken, SC), where a size-selected genomic library was sequenced using Illumina 100-bp paired-end reads. SREL identified a set of putative microsatellite loci with a known repeat motif and minimum repeat length. We tested 30 primer combinations and selected 18 loci that produced strong bands on a 1% agarose gel (Table S2). Not all loci amplified for all six species (Tables S3–S8). We used a Mastercycler Gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY) to carry out PCR amplifications with the following programme: 3 min at 94°C , 35 cycles at 1 min at 94°C , 1 min at a locus-specific temperature (Table S2), 1 min at 68.0°C and 10 min at 68.0°C . We attached fluorescent labels (HEX, FAM or TAMRA) to forward primers in combinations that allowed for multiplexing of several different loci at once. We genotyped individuals on an ABI 3730 and called alleles using GeneMarker 2.2.0 (Softgenetics, LLC., State College, PA, USA). Raw microsatellite genotype data from this study are available on Dryad.

We used Microchecker (Van Oosterhout *et al.*, 2004) to check for null alleles and other genotyping errors such as stuttering and large-allele dropout. We used Arlequin 3.5.2 (Excoffier & Lischer, 2010) to sample individual loci for deviations from Hardy–Weinberg equilibrium as well as linkage disequilibrium. We sampled each species at least once using a single collection site and host species. All six species showed significant deviations from Hardy–Weinberg equilibrium (HWE) and evidence of linkage disequilibrium at one or more sites. Because loci associated with deviations from HWE and linkage disequilibrium were not consistent across different populations of the same species, deviating loci were not excluded from the study.

Genetic clustering – local sites

We used STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) to identify genetic structure within individual collection sites for each of the six species. Simulations were performed for any collection site where seven or more total flies had been collected, and from >1 flower hosts. We ran STRUCTURE simulations of $K = 1$ to $K = 6$ clusters assuming conditions of admixture (each

individual may have recent ancestors in more than one population) and independent allele frequencies, and using each unique combination of collection site and host plant association as a 'sampling location' prior. The simulation was run 10 times for each value of K , with a burn-in length of 100 000 and 200 000 Markov Chain Monte Carlo replications. We used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to visualize log-likelihood patterns and calculate Evanno's ΔK statistic (Evanno *et al.*, 2005; Dixon *et al.*, 2013). For *B. sp21* flies in Peru and Bolivia, we performed separate runs using only flies collected from male and female *G. acuminata* flowers (omitting sympatric flies from male *G. spinulosa* flowers). This additional level of analysis was motivated by evidence of flower-specific clades of mtDNA haplotypes apparent in the *B. sp21* mtCOI tree (Fig. S6).

Genetic clustering – continental scale

Using the entire microsatellite data set for each species, we ran STRUCTURE simulations of $K = 1$ to $K = 10$ clusters (see above for parameters). We used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to investigate higher- and lower-order genetic structure in each data set. Evanno's ΔK statistic (Evanno *et al.*, 2005; Dixon *et al.*, 2013) suggested the highest order structure for all six species was at a $K = 2$. Evanno's ΔK is not as useful for revealing lower-order structure (Evanno *et al.*, 2005), so to identify additional structure in each data set, we followed the recommendation of Pritchard *et al.* (2000) and chose the K that corresponded to the moment that log-likelihood values for the data begin to level off (where additional population structure ceases to substantially increase the likelihood of the result). This lower-order structure was the focus of all subsequent analyses. We also used STRUCTURE to calculate allele frequency divergence as an estimate of genetic distance between clusters.

Results and discussion

Results of local analyses (sites in French Guiana, Peru and Bolivia) are presented in Fig. 2, and results of whole-continent analyses for each species are shown in Figs 3–8. Eight of 20 local analyses revealed some evidence of host-associated genetic structure indicating the presence of reproductive isolating barriers between sympatric flies using different hosts (Fig. 2). Continent-wide analyses showed that genetic structure within species often has a strong geographic component. Both of these patterns are described in more detail below, but because this study involves analyses of genetic structure for six independent fly species that each show different patterns, we first present and interpret results for each individual species. Names of flower hosts are abbreviated as follows: mGA = male *G. acuminata*;

fGA = female *G. acuminata*; mGS = male *G. spinulosa*; fGS = female *G. spinulosa*.

Blepharoneura sp1

Most flies in this species (>97%, 135/138) have been collected from GA flowers, mainly from male flowers (Table 1). At two sites – Villa Tunari, Bolivia, and Regina, French Guiana – *B. sp1* was also collected from other hosts: 12 specimens were collected from fGA in Villa Tunari, and near Regina, one specimen was collected from fGS. Evidence of local genetic structure was detected for *B. sp1* from Villa Tunari, Bolivia: one population included only flies collected from mGA flowers and the other population included only flies collected from fGA flowers (Fig. 2c). In the sample from near Regina, French Guiana, comparison of a singleton (one specimen) collected from an fGS flower and other flies collected from mGA flowers revealed no structure.

Continent-wide STRUCTURE analyses for *B. sp1* showed the highest level of support for a $K = 4$ (Figs 3 and S1), with genetically similar flies grouping by common patterns of host use and geography. These four clusters consisted primarily of fGA and mGA flies in Bolivia (blue cluster); mGA flies in Peru (green); one mGS fly from the Kaw Road site in French Guiana (yellow); and mGA and fGS flies from Regina site in French Guiana (red). Genetic distances calculated using microsatellite allele frequencies (Fig. 3) suggested that Peruvian and Bolivian flies are more closely related to one another than they are to French Guiana (red) flies, and that the single fly collected from mGS at the Kaw Road site in French Guiana (yellow cluster) is more closely related to mGA flies in Peru than to other flies in French Guiana. Whole-continent analyses did not split the Villa Tunari, Bolivia, flies into host-associated groups as in the local analysis, suggesting that these flies are more closely related to one another than they are to others in the data set. Microsatellite structure for this set of *B. sp1* specimens largely overlaps with relationships suggested by the mtCOI tree (Fig. S1).

Taken together, genetic structure in *B. sp1* suggests a strong role for geographic isolation in genetic differentiation, with evidence of structure evident between populations separated by both relatively long distances (>2000 km between Peru and French Guiana) and shorter distances (100's of km between Madre de Dios sites in Peru and Riberalta in Bolivia). Yet, local differentiation between the closely related flies collected from different sex flowers (fGA and mGA flowers) in Villa Tunari, Bolivia (Fig. 2C), suggests that some genetic differences may also be associated with host use (different flower sexes), at least at a local scale. The other genetically different *B. sp1* fly found on an alternative host, the single fly collected from an mGS flower in French Guiana, is genetically more similar to flies in Peru and may represent a descendent of migrants from

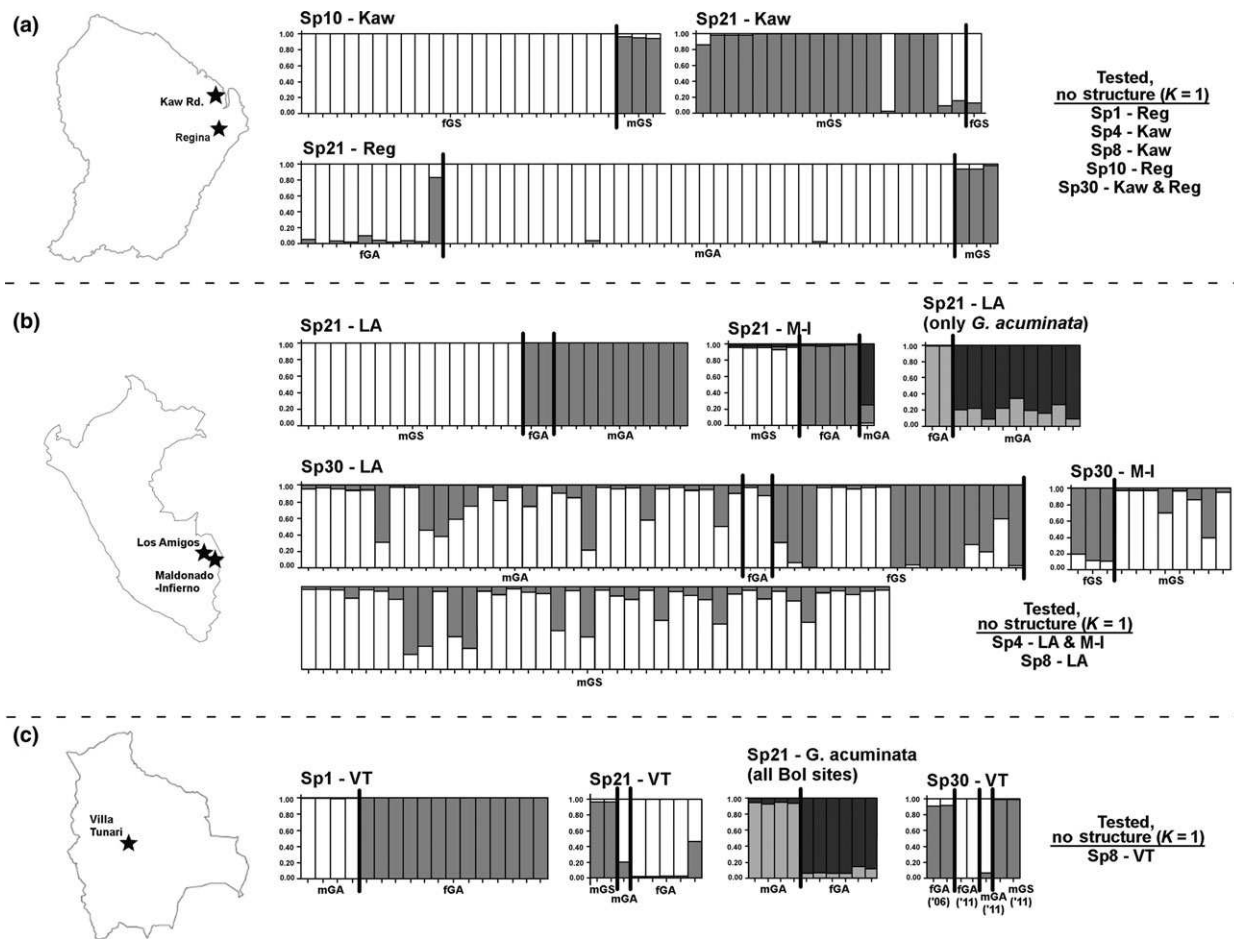


Fig. 2 Results of STRUCTURE analyses for local collection sites in (a) French Guiana, (b) Peru and (c) Bolivia where flies had been collected from two or more combinations of host plant species and tissue. Horizontal bars represent individual flies, and colours (white vs. shades of grey) represent assignment of each fly into populations based on microsatellite allele frequencies. When structure was detected, two populations ($K = 2$) were supported for most sites, although for one species at one site (*B. sp21* flies from Pto. Maldonado-Infierno, Peru) a $K = 3$ had the highest level of support. Dark horizontal lines separate groups of flies based on the host species and flower from which they were collected. For some *B. sp21* flies in Peru and Bolivia, additional STRUCTURE runs using just GA-origin flies supported two clusters, which separated flies collected from mGA and fGA flowers. Additional information regarding these and other collection sites, including site abbreviations, may be found in Table S1.

Amazonian sites. This fly has a mtCOI haplotype typical of French Guiana flies (Fig. S1), suggesting hypothetical migrants must not have been (and may not currently be) completely reproductively isolated from French Guiana flies.

Blepharoneura sp4

Although most *B. sp4* flies have been collected from mGS flowers (>93%; 254/272), a small number of flies have also been collected from fGS and mGA (Table 1). We assessed host-associated genetic structure for *B. sp4* at three local sites (Los Amigos, Peru; Pto. Maldonado-Infierno, Peru; Kaw Road, French Guiana), but detected no evidence of structure. In continent-wide

STRUCTURE analyses, a $K = 2$ had the strongest support, with resultant clusters showing a strong geographic signal: one cluster (red) was affiliated primarily with flies from the Atlantic Coastal Forest of Brazil and the Guiana Shield countries, whereas the other cluster was found primarily in Peru and Bolivia (Fig. 4). The mtCOI tree suggests a similar pattern of divergence associated with geography (Fig. S2).

Genetic structure in *B. sp4* therefore appears to be explained primarily by geographic distance, with genetic differences increasing with distance around the perimeter of South America. *Blepharoneura sp4* is a specialist on mGS flowers (Table 1), but flies are occasionally collected from other hosts (recall that apparent use of these alternative hosts is inflated in the current data

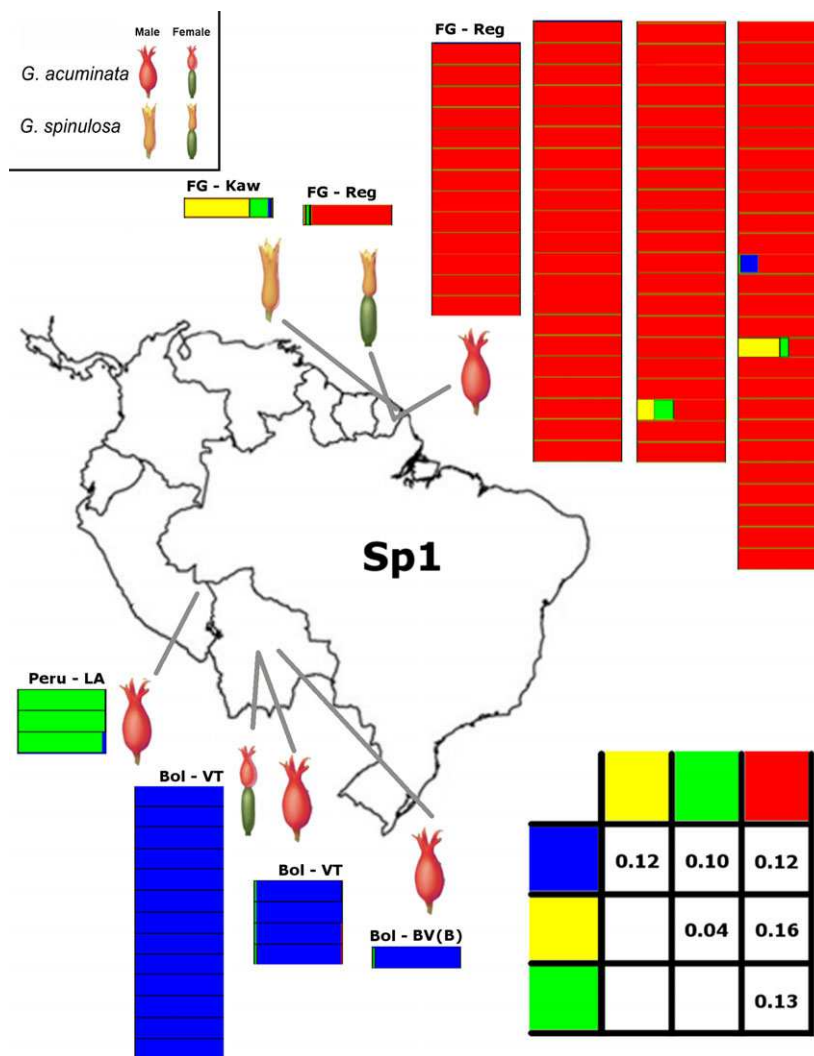


Fig. 3 Results of STRUCTURE analyses for all *B. sp1* samples. Strongest support was found for a $K = 4$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Flower hosts of each fly are explained in the key at top left. Table at bottom right shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. For details on locations of individual sites, see Table S1.

set because we intentionally scored flies that had been collected from a diverse set of hosts). Unlike in *B. sp1*, this rare variation in host use shows no associated genetic signal; changes in use of flower species or flower sex either do not persist or do not translate into reproductive isolation.

Current evidence is too limited to conclude that any *B. sp4* flies are reproductively isolated – other than by physical distance – from any others. *Blepharoneura sp4* may represent either a single species made up of widely dispersed but potentially interbreeding individuals, or two or more reproductively isolated lineages. Some behavioural and morphological evidence points to pre-mating sexual isolation as a potential reproductive barrier that may isolate allopatric flies: *Blepharoneura sp4* flies in Venezuela exhibit courtship behaviours never observed at the Napo, Ecuador site (Condon & Norrbom, 1994 [Fig. 18b], 1999). In the Napo (Ecuador), *B. sp4* exhibit courtship behaviours and sexual

dimorphism differing from other sympatric species that court and mate on the same host plant (Condon *et al.*, 2008a; Marsteller *et al.*, 2009). Further study of mating behaviour in *B. sp4* from additional different sites may be instrumental in revealing evidence of reproductive isolation between geographically distant sites.

Blepharoneura sp8

Individuals of *B. sp8* have been collected primarily from mGS flowers (>94%, 159/168), but have also been collected (rarely) from fGS, mGA and mGE (Table 1). No evidence of local genetic structure was found for *B. sp8* at any of the three sites (Bolivia, Peru and French Guiana) where it was assessed. Across the entire South American data set, however, a $K = 3$ found the strongest support. Continent-wide structure showed a strong geographic signal (Fig. 5). The three clusters approximated three groups of flies: flies from

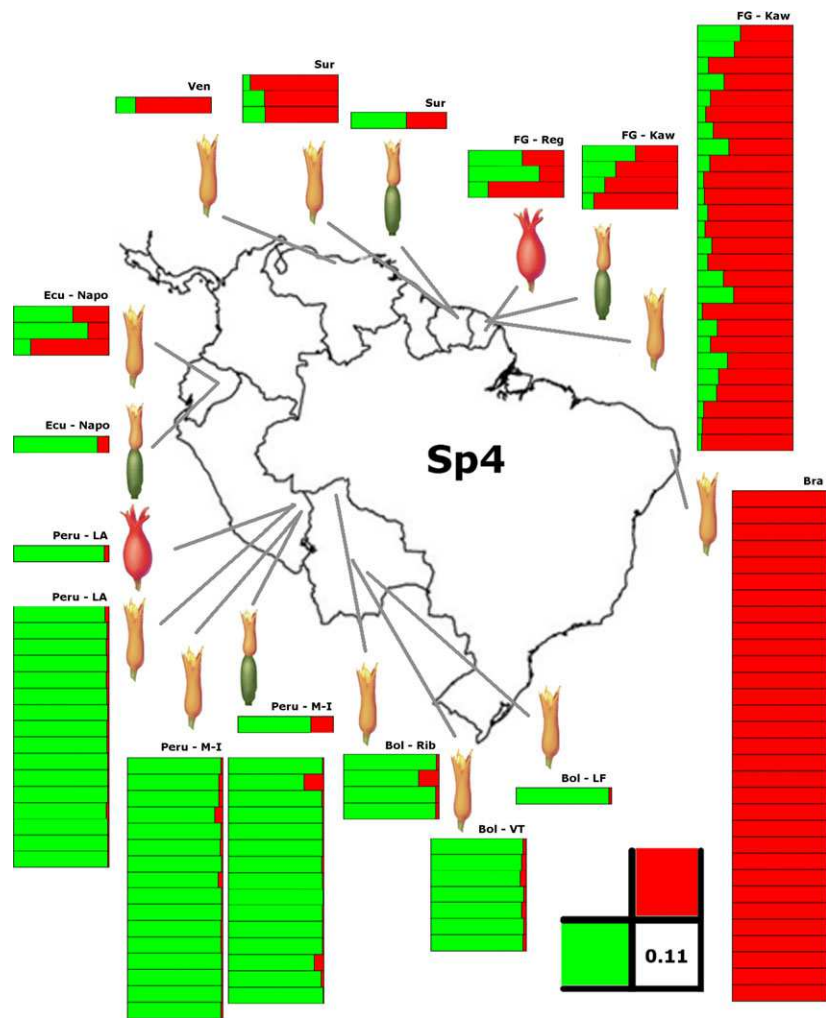


Fig. 4 Results of STRUCTURE analyses for all *B. sp4* samples. Strongest support was found for a $K = 2$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Table at bottom right shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. See Fig. 3 for a key to flower host pictures. For details on locations of individual sites, see Table S1.

mGS and from *G. eriantha* in Bolivia (blue cluster); flies collected from mGS in Peru (green); and flies from mGS and fGS in French Guiana (red). Genetic distances calculated between clusters suggest that 'Peruvian' and 'Bolivian' clusters are more closely related to one another than either is to the French Guiana cluster, suggesting that differences may be due primarily to geographic distance. Mitochondrial sequence shows a similar pattern of isolation by distance, with French Guiana flies sharing no haplotypes with flies from any other collection.

Genetic differences in *B. sp.8* appear to be associated primarily with physical distances between collections. Most *B. sp.8* flies are collected from mGS flowers (Table 1). Samples collected from non-mGS hosts show no obvious pattern of host-associated genetic differences; one possible exception revealed in the whole-continent analysis is a singleton collected from mGA from the Regina region of French Guiana. That single mGA fly appears to be more similar to mGS flies from

Los Amigos, Peru, than to sympatric individuals collected from mGS in French Guiana.

Blepharoneura sp.10

Although most individuals (>92%, 118/128) of *B. sp10* have been collected from fGS (Table 1), a few individuals have been collected from other hosts (fGA, mGS). One of two local analyses performed for *B. sp10* showed evidence of genetic structure. At the Kaw Road, French Guiana site, two clusters were supported: one included only flies collected from fGS flowers and the other included only mGS flowers (Fig. 2a). No structure was resolved at the Regina region in French Guiana, where only a single fly from a non-fGS host was available.

Continent-wide STRUCTURE analyses for *B. sp10* supported four clusters (Fig. 6), defined primarily by the following groups: flies collected from mGS in Peru (green cluster); flies collected from fGS in Venezuela

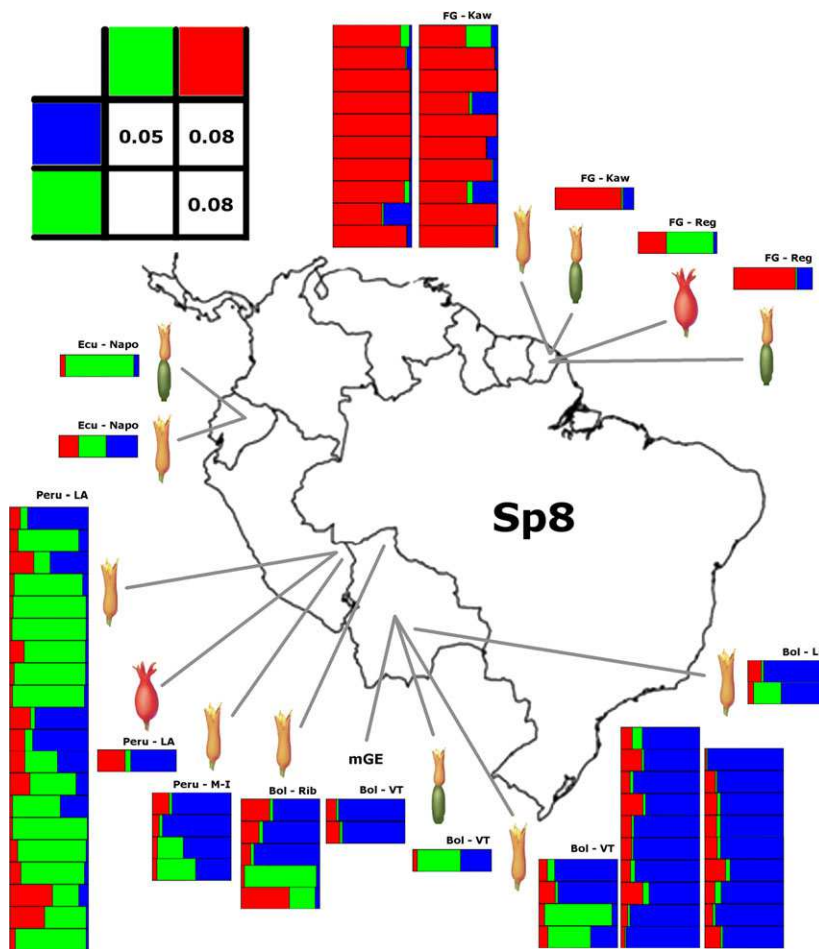


Fig. 5 Results of STRUCTURE analyses for all *B. sp8* samples. Strongest support was found for a $K = 3$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Table at top left shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. See Fig. 3 for a key to flower host pictures. For details on locations of individual sites, see Table S1.

and from sites both east and west of the Andes in Ecuador (blue); three flies collected from mGS in French Guiana (yellow); and fGS flies in French Guiana (red). Geographic and host-associated clusters did not sort into monophyletic groups on the mtCOI tree (Fig. S4), suggesting either a recent origin of genetic differences or recent / ongoing introgression. A fly collected from male *G. robusta* in Suriname affiliated primarily with the green (fGS) Peruvian cluster, whereas the single fly collected from mGS collected between Puerto Maldonado and Infierno (Madre de Dios, Peru) was more closely related to the red French Guiana flies than to other flies collected in Peru (Los Amigos, also in Madre de Dios).

Overall, *B. sp10* shows both geographic structure and host-associated structure. Most flies in French Guiana were genetically different from flies in Peru and Ecuador, particularly among flies collected from fGS, the most common host of *B. sp10*. Flies collected from hosts other than fGS clustered with flies from geographically distant sites. This was true for the three Kaw Road French Guiana mGS flies, for the two flies collected

from male *G. robusta* in Suriname and for the single Peruvian mGS fly. This finding, and the lack of monophyly between clusters in the mtCOI tree, suggests patterns of recent migration and introgression, with migrant, already-genetically differentiated flies adopting less frequently used hosts.

Blepharoneura sp.21

Flies in *B. sp21* have been collected from diverse hosts (Table 1). At each of the five sites where analyses of local differentiation were performed for *B. sp21* (French Guiana [2 locations], Peru [2 locations] and Bolivia [2 locations]), there was strong support for two to three differentiated, host-associated populations (Fig. 2). In the Regina French Guiana site, flies collected from mGS flowers clustered apart from all but one fly collected from GA flowers. At the Kaw Road, French Guiana site, two clusters were supported: one group included only mGS flies and the other included the sole fly collected from fGS flowers and three mGS flies. In both Peru and Bolivia, flies collected

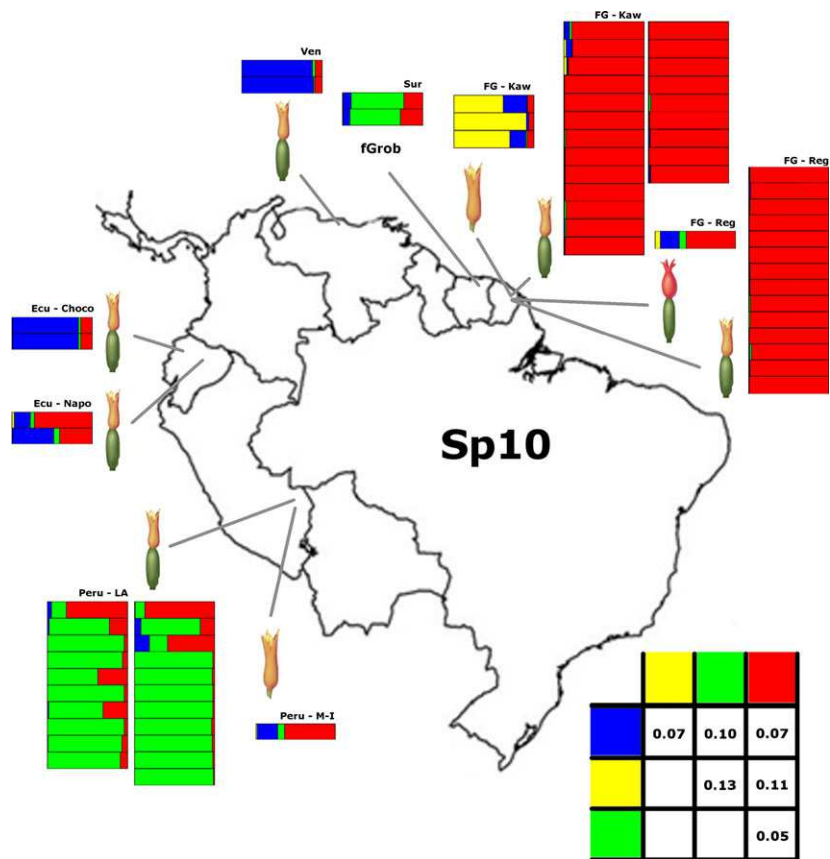


Fig. 6 Results of STRUCTURE analyses for all *B. sp10* samples. Strongest support was found for a $K = 4$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Table at bottom right shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. See Fig. 3 for a key to flower host pictures. For details on locations of individual sites, see Table S1.

from mGS were strongly differentiated from all GA-origin flies, with flies at the Puerto Maldonado-Infierno (M-I), Peru, site further splitting into mGA and fGA clusters. Due to the finding of flower sex-based structure resolved in the M-I sample, we ran separate STRUCTURE analyses for flies collected only from GA flowers in the Los Amigos, Peru site, and for the combined GA fly collection from the two Bolivian sites. These analyses supported additional genetic structure associated with flower sex: flies collected from fGA form a cluster distinct from flies collected from mGA (Fig. 2b,c).

Continent-wide STRUCTURE analyses for *B. sp21* showed highest support for four clusters (Fig. 7). In general, these represented four groups of flies: GA flies in Peru and Bolivia (blue cluster); GS flies in Peru, Bolivia and Ecuador (green); GA flies in French Guiana (red); and GS flies in French Guiana and Venezuela (yellow). Genetic distances were smallest between green and yellow clusters (GS flies) and between red and blue clusters (GA flies). A few flies in French Guiana were exceptions to this pattern, including one fly collected from fGS at the Kaw Road, French Guiana site, that affiliated more closely with the red cluster (GA) than with green or yellow clusters (GS).

Although *B. sp21* flies associated with GA and GS flowers, respectively, do not form monophyletic clades on the mtCOI gene tree (Fig. S5), the strong, continent-wide genetic structure leads us to conclude that these represent two partially or completely reproductively isolated lineages of flies, possibly even distinct species (hereafter, sp21-GA and sp21-GS). Sp21-GA and sp21-GS show no evidence of contemporary gene flow, although their divergence is sufficiently recent that their mitochondrial haplotypes do not form monophyletic clades (Fig. S5). Local analyses further suggest a more recent split between flies collected from mGA and flies collected from fGA. This split is not apparent on the continent-wide STRUCTURE analyses, but the strong differentiation between fGA and mGA flies in Peruvian and Bolivian sites (Fig. 2), along with host flower sex-associated structure on the mtCOI tree (Fig. S5), suggests sp21-GA may itself be composed of two (or more) distinct, host-associated races or incipient species, at least in Amazonia. At Los Amigos, Peru, sp21-GA and sp21-GS differ dramatically in rates of parasitism: sp21-GS suffers high mortality (>70%) due to parasitoids, but sp21-GA escapes parasitoids (Condon *et al.*, 2014). Samples from other sites (e.g. French Guiana, 2011

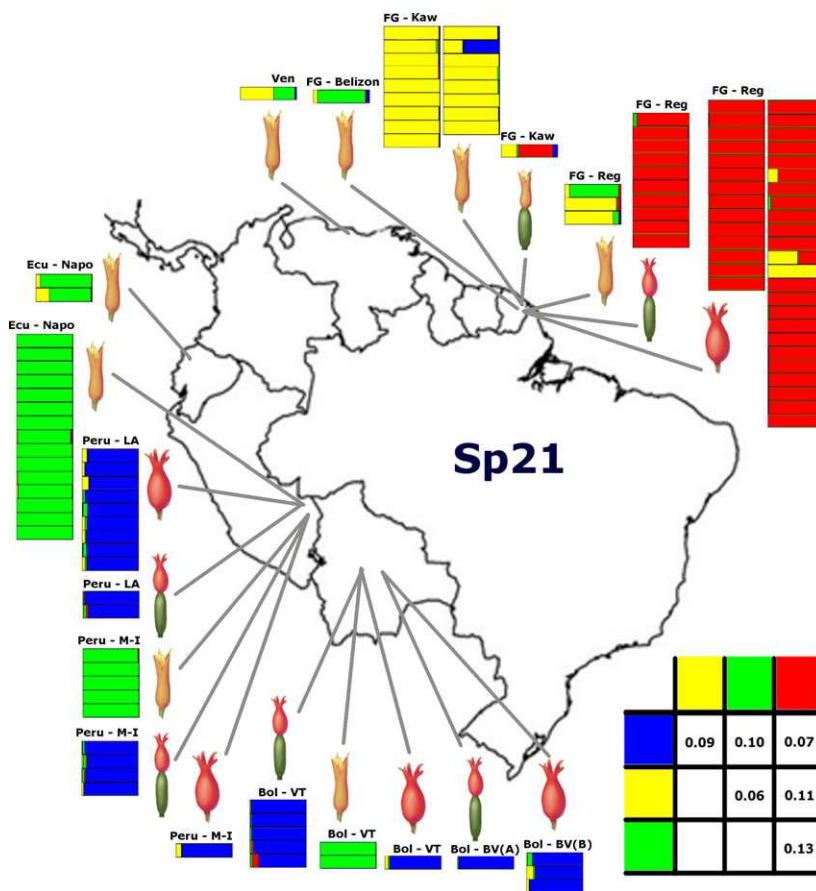


Fig. 7 Results of STRUCTURE analyses for all *B. sp21* samples. Strongest support was found for a $K = 4$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Table at bottom right shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. See Fig. 3 for a key to flower host pictures. For details on locations of individual sites, see Table S1.

collections) show similar host plant-related patterns of mortality (MA. Condon, unpublished data).

Blepharoneura sp.30

Flies in *B. sp30* are commonly collected from multiple different hosts (Table 1). Local structure was identified for *B. sp30* at three of the five sites where it was assessed (Fig. 2). At the Puerto Maldonado-Infierno (M-I), Peru site, flies grouped based on host association, with fGS and mGS flies mostly affiliating with different clusters. At the Los Amigos, Peru site, no clear pattern of host association was determined, although the highest support was for two clusters. At the Villa Tunari, Bolivia site, fly assignments to clusters apparently split along both host and temporal lines, with flies collected from fGA flowers in different years aligning with different clusters. Here also, the fGA (2) and mGA (1) flies collected in 2011 had strongly differentiated mtCOI haplotypes compared with other Bolivian flies (Fig. S6), so it may be that these represent a different subspecies of *B. sp30*. No evidence of local structure was found at the Kaw Road or Regina, French Guiana sites.

In continent-wide analyses for *B. sp30*, strongest support was found for four clusters (Fig. 8), defined

primarily by the following groups of flies: flies collected from fGS in Cacao, French Guiana (red cluster); a cosmopolitan, but primarily Amazonian cluster found across collection sites and hosts (green); some flies collected from mGS and fGS in Peru (yellow); and a few flies collected from GA flowers in Bolivia and French Guiana (blue). The pan-continental distribution of the green cluster of flies, alongside the shallow-branched, relatively low-diversity mtCOI tree (Fig. S6), suggests a recent expansion / migration of *B. sp30* flies across South America. Some host-associated genetic differences are evident from flies at local sites, but much of this may represent recent differentiation, as mtCOI haplotypes do not differ between otherwise genetically differentiated flies at sympatric sites. A possible exception (Fig. S6) to the recent expansion hypothesis is three blue cluster flies collected from GA flowers in Bolivia which had haplotypes that were 2–2.6% (10–13 bp) different in their mitochondrial sequences compared with all other flies except for one mostly blue cluster fly collected from a mGA flower in French Guiana. This may be evidence of older population structure (or a partially isolated lineage) that has been mostly replaced by the more recent expansion of the green cluster.

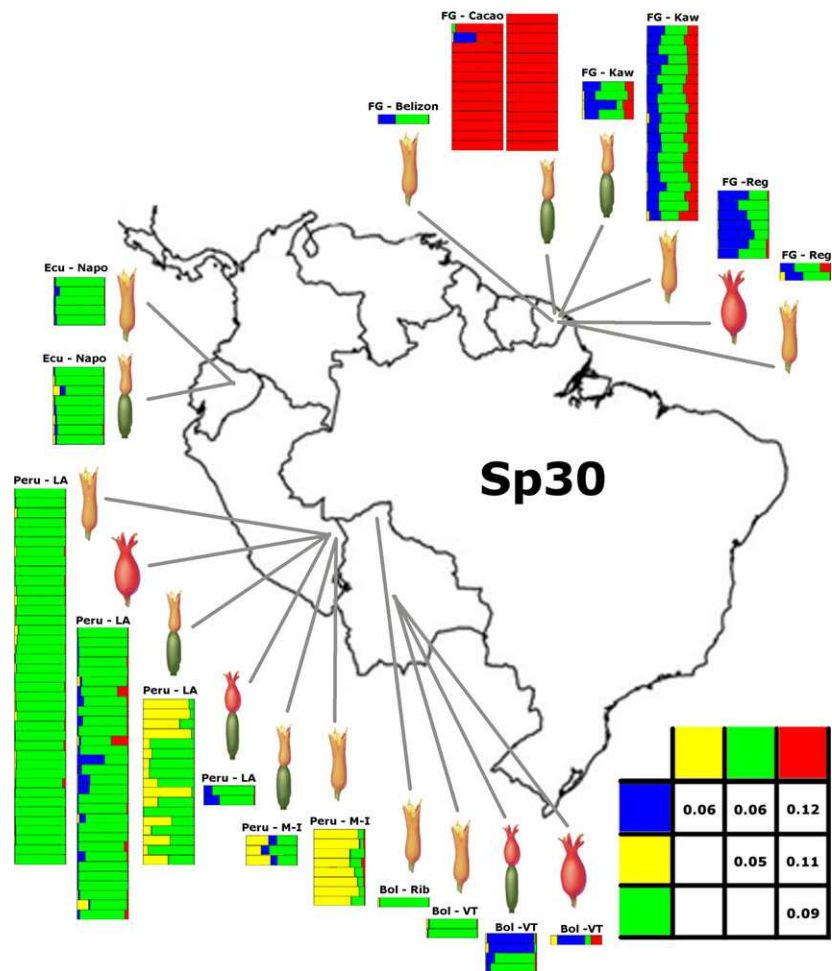


Fig. 8 Results of STRUCTURE analyses for all *B. sp30* samples. Strongest support was found for a $K = 4$. Individual flies are represented by horizontal bars, and colours of bars indicate assignment of each fly into one or more of the four different genetic clusters. Table at bottom right shows allele frequency divergence (a measure of genetic distance) between each of the four clusters. See Fig. 3 for a key to flower host pictures. For details on locations of individual sites, see Table S1.

Synthesis and conclusions

Some common themes emerge from the overall data set that may broadly explain patterns of diversity and diversification in *Blepharoneura*. First, a pattern common across all six species of *Blepharoneura* was strong genetic structure associated with geographic distance. In particular, flies collected from Brazil, French Guiana and Suriname tended to be strongly differentiated from flies in Peru and Bolivia. For species where we had also sampled from sites in Venezuela and Ecuador (e.g. *B. sp4*, *B. sp10*), these collections were either intermediate in their assignment to genetic clusters or otherwise allied with flies in one or the other two groups of sites. Isolation by distance is common among Neotropical insects (Craft *et al.*, 2010) and may reflect past histories of plant / habitat distributions or geographic barriers to fly dispersal. For *Blepharoneura*, collections represent flies and their host plants found across the South American continent, which includes 'obvious' barriers such as the Andes mountains and the Amazon and its tributaries (Hayes & Sewlal, 2004).

Second, for four of the six *Blepharoneura* species studied (*sp1*, *sp10*, *sp21* and *sp30*), sympatric sites were home to two or more genetically different fly populations that had been collected from different host species or different host-sex flowers. These flies were not isolated from one another by distance. Most locally differentiated flies are likely within one another's 'cruising range': no data on dispersal distance has been collected for *Blepharoneura*, but mark-recapture studies of other tephritid flies demonstrate dispersal distances of up to 1 km (Averill & Prokopy, 1993), and collections of *Blepharoneura* at many sites are from individual plants separated by only tens of metres (or less). Host plants are vines, and vines of different species are often intertwined (*i.e.* touching each other). Genetic structure between sympatric flies within the same species therefore strongly implies that gene flow is impeded by one or more reproductive isolating barriers.

Some specific reproductive isolating barriers that isolate sympatric *Blepharoneura* species have already been studied. Previous work suggests that there may be sexual isolation between sympatric *Blepharoneura* fly

species. Sympatric species that use the same host can differ in wing pattern, wing shape and courtship behaviours (Condon *et al.*, 2008a; Marsteller *et al.*, 2009). Courtship behaviours also differ among flies that use different species of hosts: distinctive courtship displays helped identify different sympatric species collected from the same and different hosts in French Guiana (Condon *et al.*, 2008b).

Habitat isolation, the tendency for insects to mate assortatively in different habitats (Rice & Salt, 1990; Craig *et al.*, 1993; Funk *et al.*, 2002), may also contribute to reproductive isolation in *Blepharoneura*. Among the sexually dimorphic host plants of *Blepharoneura*, male inflorescences are borne on actively climbing leafy branches, whereas female flowers are borne on pendulous leafless branches. Plants of different sexes present quite different visual cues to the flies and may also differ in olfactory cues; in some cucurbits, male and female flowers produce different volatile compounds (Theis *et al.*, 2009). As in other tephritid flies (e.g. Forbes & Feder, 2006), *Blepharoneura* may use olfactory or visual cues associated with male and female flowers of the same or different *Gurania* species to discriminate between hosts. Several species of *Blepharoneura* are known to court and mate on the surface of their host plants (Condon & Norrbom, 1999; Condon *et al.*, 2008a) where they actively abrade leaf surfaces (Driscoll & Condon, 1994), which could release volatile compounds. If flies have behavioural preferences for different host plants, these behaviours likely translate into assortative mating and contribute to reproductive isolation. Future work should focus on understanding both mate choice and host plant choice in *Blepharoneura*.

When local and continent-wide analyses are taken together, a third important pattern emerges from the four species (sp1, sp10, sp21 and sp30) that showed some evidence of local host-associated genetic structure (Fig. 2): differentiated, sympatric flies using different hosts were usually not one another's closest relatives (Figs 3, 6, 7 and 8). Current differences in host use may therefore arise primarily after flies from different regions come into secondary contact. Synthesis of results from across species and spatial scales suggests that geographically isolated flies may indeed be evolving reproductive isolating barriers, such that when they come into contact, selection against hybrids favours traits that reduce opportunities for mating between previously isolated groups. As such, use of different host tissues and any subsequent habitat isolation may be the result of reinforcement (e.g. Servedio & Noor, 2003), with selection against potentially low-fitness hybrids favouring differentiation in host plant use or in characters related to mate recognition and acceptance. One apparent exception to the overall pattern of locally differentiated flies having closer relatives elsewhere were the *B. sp1* flies from Villa Tunari,

Bolivia, which formed two local clusters based on sex of host flower (Fig. 2c) and were also apparently one another's closest relatives (Fig. S2).

Knowledge gained from microsatellites suggests new hypotheses regarding the origins of reproductive isolation and diversity in *Blepharoneura*. One hypothesis emerges from previous research showing that lethal parasitoids of *Blepharoneura* can be highly host plant and flower sex specific (Condon *et al.*, 2014): selection may favour host plant shifts by flies when and where specific lethal parasitoids are abundant. If use of different hosts results in assortative mating (as it does in other tephritids: Craig *et al.*, 1993; Feder *et al.*, 1994), habitat isolation may often be the result of parasitoid-mediated host shifts. Escape from parasitoids may also be mediated by bacterial symbionts such as *Wolbachia*, which themselves can cause reproductive isolation between lineages of insects carrying different strains (Stouthamer *et al.*, 1999; Bordenstein *et al.*, 2001). Future work will explore far denser population genomic marker systems that allow us to model current and historical gene flow for these flies and parallel population genomic analyses of the *Bellopius* parasitoid wasps that may play a major role in *Blepharoneura* diversity (Condon *et al.*, 2014).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Collection information for *Blepharoneura* flies used in this study.

Table S2 Eighteen primer pairs use to amplify microsatellite loci for six species of *Blepharoneura* flies.

Table S3 Summary of eleven microsatellite loci genotyped for 104 *Blepharoneura* sp1 flies used in this study.

Table S4 Summary of 11 microsatellite loci genotyped for 134 *Blepharoneura* sp4 flies used in this study.

Table S5 Summary of 12 microsatellite loci genotyped for 83 *Blepharoneura* sp8 flies used in this study.

Table S6 Summary of 13 microsatellite loci genotyped for 70 *Blepharoneura* sp10 flies used in this study.

Table S7 Summary of 14 microsatellite loci genotyped for 121 *Blepharoneura* sp21 flies used in this study.

Table S8 Summary of 18 microsatellite loci genotyped for 181 *Blepharoneura* sp30 flies used in this study.

Figure S1 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp1 flies used in this study.

Figure S2 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp4 flies used in this study.

Figure S3 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp8 flies used in this study.

Figure S4 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp10 flies used in this study.

Figure S5 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp21 flies used in this study.

Figure S6 Neighbor-joining tree of mitochondrial COI sequences from the *Blepharoneura* sp30 flies used in this study.

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