Ecological Entomology (2009), **34**, 66–73 DOI: 10.1111/j.1365-2311.2008.01047.x

Six cryptic species on a single species of host plant: morphometric evidence for possible reproductive character displacement

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Abstract. 1. Diversification of some highly host-specific herbivorous insects may occur in allopatry, without shifts in host use. Such allopatric divergence may be accelerated by sexual selection operating on courtship displays. Wing size and shape may affect visual and vibrational courtship displays in tephritid fruit flies. Geometric morphometric methods were used to examine wings of six sympatric cryptic species in the neotropical genus *Blepharoneura*. All six species feed on flowers of the same species of host (*Gurania spinulosa*), a neotropical vine in the Cucurbitaceae. Three of the fly species court and mate in close proximity on the host. Thus, courtship behaviours could serve as important reproductive isolating mechanisms. Two sets of hypotheses were tested: (i) species differ in wing shape and wing size; and (ii) species are sexually dimorphic in wing size and wing shape. Wing size differed among a few species, but wing shape differed significantly among all six species. Sexual dimorphism in wing size was found in only one species, but sexual dimorphism in wing shape was found in two of the three species known to court on the same host plant. In the two sexually dimorphic species, wing shape differed among males, but not among females. This suggests that selection for reproductive character displacement might accelerate divergence in wing shape.

Key words. Courtship, Diptera, flies, host specificity, neotropics, sexual selection, speciation, Tephritidae, tropical diversity, wing shape.

Introduction

Phytophagous insects make up more than a third of all described organisms on Earth (Strong *et al.*, 1984; Wilson, 1992). Although many attempts to explain this extraordinary diversification focus on patterns of host use (Ehrlich & Raven, 1964; Mitter *et al.*, 1991; Novotny *et al.*, 2006; Nyman *et al.*, 2006), recent evidence suggests that the most rapid rates of evolution are caused by sexual selection (Arnqvist *et al.*, 2000; Mendelson & Shaw, 2005). In many insects, mate choice follows elaborate sexually-selected courtship displays (Thornhill & Alcock, 1983; Choe & Crespi, 1997). Among insects that court and mate exclusively on their hosts, both shifts in host use and sexual selection on courtship displays can promote divergence of host-specific populations (Faust & Brown, 1998; Rodriguez *et al.*, 2004; Cocroft & Rodriguez, 2005). Courtship displays can be directly affected by host plants: vibrational courtship signals can be transmitted differently by the same tissues of different plants, or by different tissues of the same plant (Sattman & Cocroft, 2003; Cocroft *et al.*, 2006). Thus, shift in use of either host plant species or host plant part could result in divergence of courtship-related traits.

Lineages diverging as a result of behavioural changes (e.g. host shifts, courtship displays, or both) are often morphologically cryptic (Bush, 1969; Wood, 1980; Wood & Keese, 1990; Via, 1991; Wells & Henry, 1998; Emelianov *et al.*, 2002; Bickford, 2006). The family of true fruit flies (Tephritidae) includes classic examples of morphologically cryptic host races (e.g. *Rhagoletis*: Bush, 1969; Berlocher, 2000; Filchak *et al.*, 2000; *Eurosta*: Waring *et al.*, 1990; Craig *et al.*, 1993). These well-known examples all involve shifts in use of host taxa both

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as sites for egg laying and courtship and mating. In another tephritid genus, *Blepharoneura*, diversification may involve shifts in use of both host taxa and host parts (Condon & Steck, 1997). However, much diversification in *Blepharoneura* may occur in allopatry without any host shifts, perhaps accelerated by sexual selection (Condon *et al.*, 2008a, b).

Courtship displays of *Blepharoneura* are elaborate and often occur on the surfaces of host plants (Condon & Norrbom, 1999; Condon *et al.*, 2008a). Some displays of *Blepharoneura* involve extremely rapid wing motions that could cause vibrations that could be transmitted by plant tissue. Other displays involve slower wing motions during which wings of males are rotated and outstretched while males face females. In some species, males display in leks on the surfaces of leaves of the host plants where adult females often graze (Condon & Norrbom, 1994, 1999). In these situations, sexual selection could cause rapid evolution of male display characters and accelerate allopatric speciation (Mendelson & Shaw, 2005). In species that occur sympatrically on the same host, selection for reproductive character displacement may cause further divergence of courtship displays.

Multiple sympatric cryptic species of *Blepharoneura* often infest single species of sexually dimorphic and functionally dioecious host plants (Condon & Norrbom, 1994; Condon & Steck, 1997). In eastern Ecuador, six sympatric cryptic species of *Blepharoneura* infest calyces of flowers of a single species of host plant – *Gurania spinulosa* Cogn. (Condon *et al.*, 2008a, b). Two out of the six species lay eggs exclusively on female flowers, a third lays eggs on both male and female flowers, and the remaining three species are specialists on male flowers. Although courtship of species feeding (as larvae) on female flowers has not been observed in the field, the courtship of all three male-flower specific species has been observed in the field on a single individual host plant bearing male flowers (Condon *et al.*, 2008a). Species infesting *G. spinulosa* flowers are almost indistinguishable morphologically; however, substantial inter-specific differences (7–10%) in nucleotide sequence in the mitochondrial gene cytochrome oxidase subunit I clearly distinguish species (Condon *et al.*, 2008a, b). If species that court and mate on the same host surfaces use similar cues during courtship, inter-specific matings – even among distantly related species – could occur. If fitness of hybrids is low (or if post-mating isolation is complete), there should be strong selection for reproductive character displacement (Coyne & Orr, 2004), particularly in species encountering similar species on the same host plants. Indeed, courtship displays of these sympatric species of *Blepharoneura* differ noticeably in speed, form, and tempo (Condon *et al.*, 2008a).

Courtship displays involving elaborate movements of wings bearing intricate pigmentation patterns, could involve visual cues (Edwards *et al.*, 2007), which may be affected by wing shape or size. Displays involving vibrational signals may also be affected by wing size and shape (Sivinski & Dodson, 1992). If wing shape affects displays, shape should differ among these sympatric species, especially among species that court and mate on the same surfaces. Sexual selection can accelerate evolution and is associated with speciation in diverse groups (Andersson, 1994; Mendelson & Shaw, 2005) including remarkably species-rich groups such as the Hawaiian *Drosophila* (Kaneshiro, 1988), which – like *Blepharoneura* – engage in elaborate courtship displays and have elaborately patterned wings.

Significant sexual dimorphism is often a signature of sexual selection (Andersson, 1994). If selection for reproductive character displacement also occurs, selection could favour divergence of sexually selected characters in species that court and mate in the same locations. If wing shape affects male displays in *Blepharoneura*, species that court and mate in the same locations should differ in wing shape. To determine whether sympatric cryptic species of *Blepharoneura* in Ecuador differ in wing shape or size, landmark-based geometric morphometric methods (Rohlf & Marcus, 1993; Adams *et al.*, 2004) were used to test two sets of hypotheses: (i) species differ in wing shape and wing size, and (ii) species are sexually dimorphic in wing size and wing shape.

Methods

Wings of voucher specimens of six species of *Blepharoneura* identified through phylogenetic analysis of the sequence of cytochrome oxidase subunit I (COI), a mitochondrial gene (Condon *et al.*, 2008a, b) were examined. Species status of these lineages has been corroborated through analysis of two nuclear genes – EF1-α and CAD (Condon *et al.*, 2008b). All specimens were collected within a radius of 8 km from the Jatun Sacha Biological Station, which is located near Misahuallí in the Napo province of eastern Ecuador: 01°03.941S, 77°36.998W (Condon *et al.*, 2008a, b). For wing shape analyses, only specimens reared from mature flowers were included, because flies complete their development in mature flowers (not immature flowers) in nature. All of these are newly discovered and undescribed species (without scientific names). Thus, these lineages have either been given monikers related to the species' sites and styles of courtship display (e.g. clapleaf, shiverer) or have been labelled with the specific epithets of closely-related species (e.g. atomaria, perkinsi) (Table 1).

To prepare slide-mounted wings, the right wing of each fly was removed. Wings were boiled in a dilute potassium hydroxide (KOH) solution, rinsed, and mounted in Euparal on a glass slide. As Euparal-mounted wings are permanently mounted, we could not take multiple repeated measurements to estimate the error associated with variation in compression and flattening of the wings. If the right wing was damaged, the left wing was used: preliminary analyses showed no significant difference in shape between left and right wings (D. C. Adams, unpubl. data). Images were captured with a FUJI FinePix S2 Pro digital camera (resolution 4256 × 2848) mounted on a WILD3Z dissecting microscope.

Wing shape was quantified using landmark-based geometric morphometric methods (Rohlf & Marcus, 1993; Adams *et al.*, 2004). These methods allow a rigorous quantification of wing shape after the effects of non-shape variation have been mathematically held constant. For this approach, a series of biological landmarks are identified on each wing mount image, and are used for the subsequent quantification of shape (Fig. 1). First, the x, y coordinates of 14 landmarks were digitised on wing

Table 1. Six sympatric species of *Blepharoneura* feed as larvae on flowers of *Gurania spinulosa* in eastern Ecuador. Courtship display location determined through observation of a single individual male *Gurania spinulosa* (Condon *et al.*, 2008a). Clades are identified by letters used to label clades revealed through phylogenetic analysis of mtDNA COI sequence (Condon *et al.*, 2008a) and by numbers denoting the same clades revealed through phylogenetic analysis of mtDNA COI and nuclear genes EF1-α and CAD (Condon *et al.*, 2008b).

Moniker	Larval host part	Courtship display location (on male *G. spinulosa*)	Clade
Atomaria	Male flowers	Male inflorescence and leaves	A, sp4
Clapleaf	Male flowers	Leaf–distinctive display	B, sp8
Flatclap	Female flowers	Unknown (not on male plant)	E, sp11
Perkinsi	Female flowers	Unknown (not on male plant)	F, sp10
Shimmyleaf	Male flowers	Leaf–distinctive display	D, sp12
Shiverer	Male and female flowers	Unknown (reared from plant where behaviors observed, but no courting individuals discovered)	C, sp30

mount images, using tpsDIG (Rohlf, 2004a). Next, the landmark coordinates for all specimens were aligned using a generalised Procrustes analysis (GPA; Rohlf & Slice, 1990), which scales, centres, and rotates landmark configurations using a least squares criterion. After GPA, the resulting configurations are invariant with respect to size, position, and orientation (i.e. they only vary by shape). From the aligned specimens, shape variables were then generated as partial warp scores from the thin-plate spline (Bookstein, 1991) and the standard uniform components (Rohlf & Bookstein, 2003). These variables can be used in analyses of shape variation or covariation with other variables (e.g. Adams, 2004; Adams *et al.*, 2007). Principal component analysis (PCA) was used to visualise shape variation among groups. GPA, TPS, and PCA were performed with tpsRELW (Rohlf, 2004b).

Using the above procedure, wing shapes of 104 individual specimens were quantified. A permutation method was used to test for differences among species-sex groups for both wing size and shape. Wing size was measured as centroid size (CS), which is calculated as the square root of summed squared distances between landmarks of a specimen and its centroid (Bookstein, 1991). Shape was defined by the $2k-4$ shape variables possible from k landmarks (in this case, 14 landmarks leads to 24 shape variables; see Adams *et al.*, 2004). Average CS and shape were calculated for the 12 observed species-sex groups. Individual values were subsequently randomly assigned to the 12 possible groups and average values were calculated for 9999 random permutations. In every random permutation, plus the observed case, the Euclidean distances (D) among means (for CS, this is equivalent to the absolute differences between means) were calculated. The significance of observed values was ascertained as the empirical probability of finding an equal or greater value from distributions of 10000 random values. Any observed value greater than the value of the 95th percentile (i.e. $P < 0.05$) from the random distribution was considered to be indicative of a significant difference. Finally, to assess specific differences in sexual dimorphism, the shape differences between males and females were expressed as a vector, and the angular difference in orientation between sexual dimorphism vectors for pairs of species was calculated (for details see Collyer & Adams, 2007).

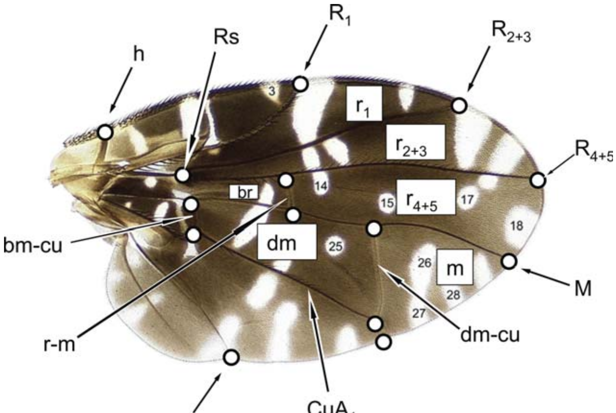

Fig. 1. Image of wing used for collection of morphometric data. The 14 landmarks used in this study are shown as open dots. Relevant wing cells are labelled (in boxes), and wing veins are labelled with arrows. Wing spots useful in identifying these species are also labelled (numbers).

Results

Wing size revealed a few inter-specific differences and only one instance of sexual dimorphism. Significant differences were found between two of the four species that infest male flowers: wings of clapleaf females were significantly larger than shiverer females ($CS_{clapleaf} = 6.49$; $CS_{shiverer} = 5.73$; $P = 0.0017$), and males of these two species showed a similar pattern ($CS_{clapleaf} = 6.24$; $CS_{shiverer} = 5.48$; $P = 0.0048$). Wings of males of male-flower-specific clapleaf were also significantly larger than wings of males of female-flower-specific perkinsi ($CS_{perkinsi} = 5.43$; $P = 0.0010$). Among the three species infesting female flowers, flatclap males had significantly larger wings than males of either of the other two species (shiverer or perkinsi) ($CS_{flatclap} = 6.42$; $P = 0.0016$ and $P = 0.0006$ respectively). When sexual dimorphism within species was examined, only one species, perkinsi, exhibited significant wing size dimorphism, with females having larger wings than males

($CS_{\mathrm{Male}} = 5.43$; $CS_{\mathrm{Female}} = 6.19$; $P = 0.0016$). Thus, wing size patterns revealed some species-level differentiation, particularly among males of species infesting the same gender flower, but revealed only one instance of sexual size dimorphism.

When wing shape was examined, even more interesting patterns were exposed. For instance, between-species comparisons revealed considerable species-specific wing shape differences. For males, all 15 pairwise comparisons between species were significant, implying that each species is characterised by a distinctive male wing shape (Table 2). In addition, 13 out of 15 comparisons between females were significant (Table 2). The only inter-specific comparisons of female wing shape that were not significant were atomaria versus clapleaf (both of which infest male flowers), and flatclap versus perkinsi (both of which infest female flowers).

When patterns of wing shape variation within species were examined, most species did not exhibit significant sexual dimorphism. However, significant sexual dimorphism in wing shape was revealed for two species: clapleaf ($D_{\mathrm{M–F}} = 0.0217$; $P = 0.0029$) and atomaria ($D_{\mathrm{M–F}} = 0.0151$; $P = 0.0372$), neither of which was sexually dimorphic in wing size. Interestingly, females of these species were not different in wing shape, but males did differ in wing shape (Table 2). Therefore, not only did these species exhibit significant sexual dimorphism, but they also had differing patterns of sexual dimorphism, where males became more divergent relative to females.

A PCA on all specimens was performed to visualise patterns of shape variation (i.e. relative warps analysis). When species × sex wing shape means were plotted along the first two principal components of shape, statistical patterns were visually confirmed (Fig. 2). When viewed along PC1, wing shapes varied from relatively elongated wings towards the positive side of PC1, to relatively compressed wings towards the negative side of PC1. In addition, changes along PC1 corresponded to changes in the shape of wing cell r_{4+5} (Fig. 2). Species-specific differences aligned mostly along PC1 (Fig. 2), where perkinsi and flatclap exhibited relatively longer wings than the remaining four species. The exception to this pattern was shimmyleaf, which was differentiated from the remaining species along PC2. Wing shapes of this species appeared relatively shorter in the basal radial (br) wing cell. Shimmyleaf is one of the three species that have been observed courting and mating on a single male individual of *G. spinulosa*.

Our statistical findings for sexual dimorphism in wing shape were also visually confirmed using PCA. In particular, females of atomaria and clapleaf were found in close proximity in this plot, signifying that they were similar in wing shape (Fig. 2B,C). However, males of these species were found in different locations in shape space from one another and from their respective females, confirming the existence of sexual dimorphism in these species. In addition, the sexual dimorphism vectors for these species appeared to be oriented in different directions: the differences between the sexes in atomaria were found primarily along PC1, but differences between the sexes in clapleaf were most evident along PC2. This signified that patterns of sexual dimorphism for these species was divergent, and based largely on male differences. Atomaria and clapleaf are two of the three species that have been observed courting and mating on the same individual host plant as shimmyleaf (Condon *et al.*, 2008a).

To quantify the differences in sexual dimorphism between atomaria and clapleaf, the angle between sexual dimorphism vectors of atomaria and clapleaf was calculated. Their orientation differed by 60.5°, confirming the divergent patterns of sexual dimorphism in these species (atomaria versus clapleaf). When viewed as deformation grids (Fig. 3), the difference between males in these sexually dimorphic species was most evident in the central region of the wing, where the distance between r–m and Rs to bm–cu is longer in clapleaf relative to atomaria. The two also differ noticeably in the relative lengths of r_{4+5} (atomaria is longer) and in the shape of r_{2+3}. In clapleaf, r_{2+3} is distinctly triangular and broader at the distal edge of the wing than atomaria, which has a bend in the proximal portion of at the intersection with r–m.

Discussion

Significant inter-specific differences in wing shape of males were found among all six sympatric cryptic species of *Blepharoneura* that infest flowers of the same host species in eastern Ecuador. Three of the species feed exclusively on male flowers (atomaria, clapleaf, shimmyleaf), two feed exclusively on female flowers (perkinsi, flatclap), and the sixth (shiverer) feeds on both male and female flowers. All three of the species specific to male flowers court and mate on their larval host, and have even been seen courting and mating on the same individual host (Condon *et al.*, 2008a). Given such extreme spatial and temporal overlap in the locations of courtship arenas, it is likely that courtship displays function as important reproductive isolating mechanisms, and that wing shape may evolve in response to selection on courtship displays.

Table 2. Pairwise species differences in shape for females (below diagonal) and males (above diagonal and grey cells). Values in bold indicate no significant difference in shape based on a permutation procedure ($P > 0.05$).

	Atomaria	Clapleaf	Flatclap	Perkinsi	Shimmyleaf	Shiverer
Atomaria	------	0.0245	0.0217	0.0270	0.0263	0.0199
Clapleaf	**0.0135**	------	0.0277	0.0312	0.0358	0.0256
Flatclap	0.0281	0.0290	------	0.0210	0.0317	0.0254
Perkinsi	0.0243	0.0271	**0.0218**	------	0.0413	0.0294
Shimmyleaf	0.0297	0.0350	0.0428	0.0427	------	0.0385
Shiverer	0.0208	0.0187	0.0268	0.0327	0.0415	------

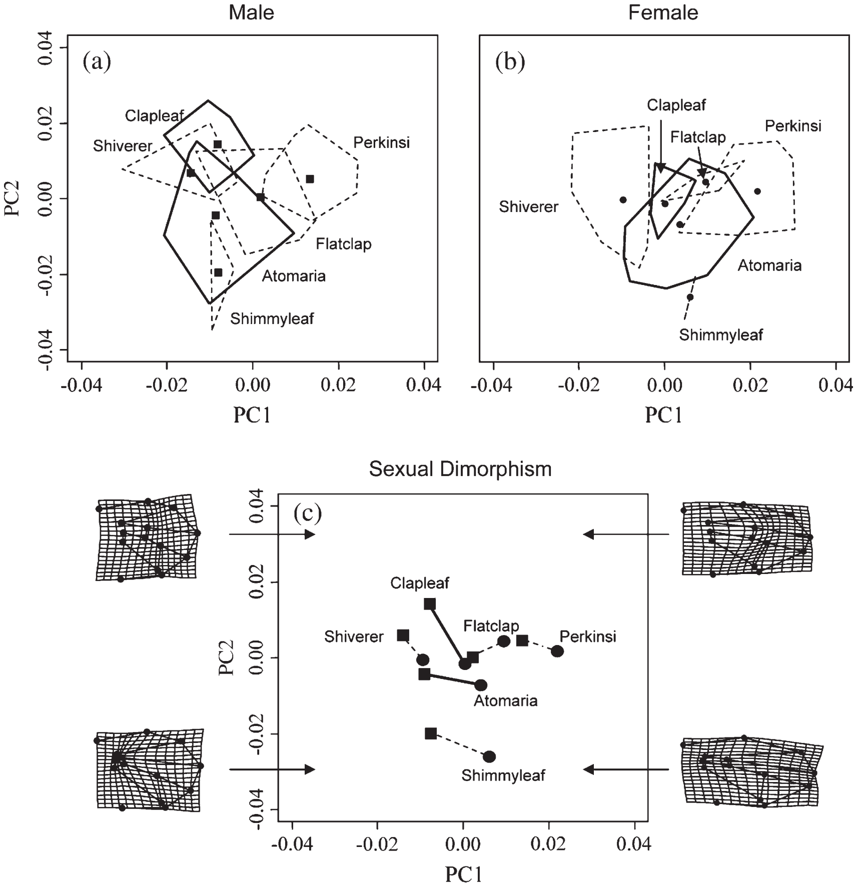

Fig. 2. Principal components (PC) analysis of all specimens (i.e. relative warps). In plots, group means and convex hulls are displayed. In each plot, the first two PCs (accounting for 39.9% of the overall shape variation) are shown: PC1 = 21.16%, PC2 = 18.75%. Solid lines (a, b) and vectors (c) indicate species exhibiting significant sexual dimorphism. a and b) male and female means and convex hulls are illustrated, respectively. c) male (square) and female (circle) means are displayed connected by vectors representing sexual dimorphism. Sample sizes: atomaria (12♂, 9♀), clapleaf (9♂, 11♀), flatclap (6♂, 4♀), shiverer (9♂, 11♀), shimmyleaf (3♂, 2♀). Thin-plate spline deformation grids, shown to indicate patterns of variation in shape space, have been accentuated by a factor of three to enhance visual interpretation.

Courtship behaviours of many tephritids involve either elaborate wing displays (Headrick & Goeden, 1994), acoustic vibrational signals (Webb *et al.*, 1983, 1984; Sivinski *et al.*, 1984; Sivinski & Webb, 1985; Alonso-Pimentel *et al.*, 2000) or both (Sivinski *et al.*, 1999). Courtship displays of these species of *Blepharoneura* involve elaborate wing motions (Condon & Norrbom, 1999; Condon *et al.*, 2008a). Most species incorporate a type of semaphoring, during which the wing is rotated and held outstretched (with the plane of the wing perpendicular to the substrate) while the male stands or paces in front of the female. Such displays may provide visual cues (e.g. wing spot patterns; Condon & Norrbom, 1994; Condon *et al.*, 2008a) that could be affected by wing shape.

Other wing movements repeated during courtship displays of *Blepharoneura* are extremely rapid. To freeze wing motion, high-speed video requires a frame rate of 1000 f/s with 1/4000 s shutter speed (M. Condon, unpubl. data). In all species that have been observed, these displays are performed while flies are standing on the surfaces of the plants. In some of the species (e.g. shiverer), rapid wing movements during courtship are also accompanied by rhythmic contact between the abdomen and the wings. All these movements would generate vibrations that could travel through the plant tissue and be detected by potential mates (Cocroft & Rodriguez, 2005; Cocroft *et al.*, 2006). Wing shape could affect the frequency and form of the signals used during courtship.

The finding that wing shape differs among males of all species, supports our hypothesis that wings – either through their involvement in visual displays or acoustic displays – are used in courtship, which functions as an important pre-mating reproductive isolating mechanism. As all the species in our study are sympatric, and oviposit in the same species of host, they may share a history of selection on characters affecting reproductive isolation. For most species, patterns of divergence in wing shape appear to be similar for both males and females: evidence of sexual dimorphism was not found within most species. Only one species (perkinsi) was sexually dimorphic in wing size: males are smaller than females, as in many other species of tephritids (Sivinski & Dodson, 1992). As courtship displays of perkinsi and the other two species infesting female flowers have not yet been observed in the field, the relationship between sexual dimorphism and selection for reproductive character displacement in these female-flower infesting species cannot currently be assessed.

Three of the species that infest male flowers are known to court and mate in the same location (Condon *et al.*, 2008a). Sexual dimorphism in wing shape (but not wing size) was discovered in two of the three species that have been observed courting on the surface of the same individual host plant. Both atomaria and clapleaf are sexually dimorphic: shapes of males' wings differ significantly, but shapes of females' wings do not

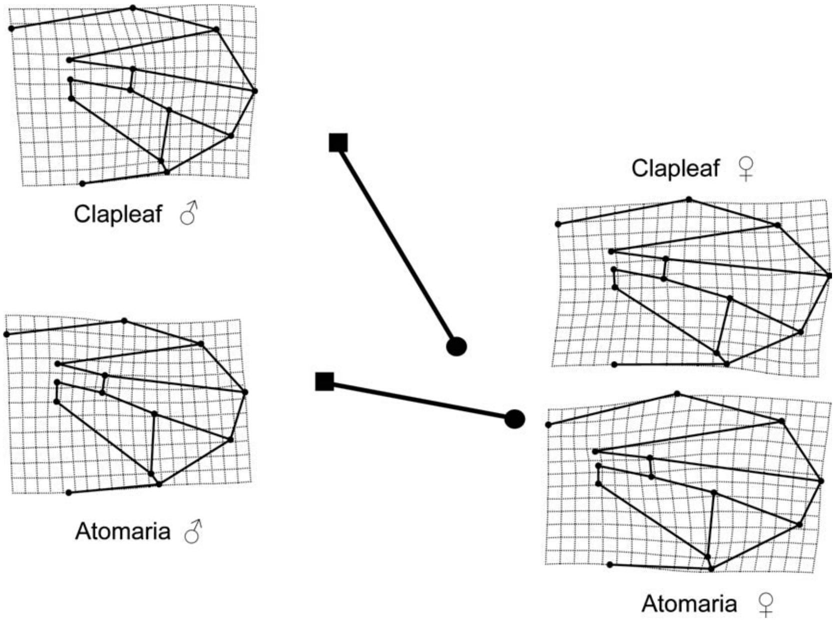

Fig. 3. Sexual dimorphism of wing shape shown as thin-plate spline deformations. Vectors describing wing shape differences between males and females are as presented in Fig. 2, but thin-plate spline deformation grids have been accentuated by a factor of five to enhance visual interpretation. Deformation grids for females are relative to the overall average specimen, and deformation grids for males are relative to females of the same species.

differ. Molecular phylogenetic analyses show that atomaria and clapleaf are more closely related to each other than to the other species (shimmyleaf) that courts on male plants (Condon *et al.*, 2008b). Furthermore, our morphometric analyses show that wing shapes of both males and females of shimmyleaf differ significantly from all other species. If wing shape affects courtship displays and mate choice, one would expect that selection for reproductive character displacement would be strongest between species with the most similar wing shapes, especially if those species display in courtship arenas that overlap in space (i.e. occur on leaves on the same individual plants).

Although these species are recently discovered and morphologically similar, available molecular evidence suggests that these six species diverged at least 3 million years ago, probably in allopatry, not in sympatry (Condon *et al.*, 2008b). In isolated populations, sexual selection can lead to rapid evolution of distinctive courtship displays (Mendelson & Shaw, 2005). Thus, sexual selection can lead to rapid divergence of allopatric populations, without any changes in patterns of host use. Indeed, in *Blepharoneura*, patterns of host use appear to be highly conserved within lineages. If divergent populations rejoined (perhaps as a consequence of habitat expansion), two species that overlap in patterns of host use (and courtship arenas) would come into sympatry. Selection for reproductive character displacement could follow, and might be most intense on male characters affecting female choice. In such circumstances, sexual dimorphism would result if there is no strong genetic correlation between the sexes for the trait under selection.

Selection for reproductive character displacement is expected to be most intense on closely related species (such as atomaria and clapleaf) that court and mate in the same locations (e.g. in the same places on the same individual plants). Further, sexual dimorphism should be most pronounced where the two species are found in sympatry, and less pronounced in regions where the species do not overlap. These predictions suggest a direct link between behavioural displays exhibited by males of different species, the degree of wing shape dimorphism, and the extent of reproductive isolation. If these predictions are correct, they suggest an explicit model for *Blepharoneura* diversification. Sexual selection acts on differences in behavioural courtship signals, generates differential selective pressures on morphological characteristics of males, and results in morphological divergence in wing characteristics affecting courtship displays. Sexual selection on courtship displays would therefore accelerate lineage diversification and radiation. Thus, similar to models proposed to explain the stages of vertebrate adaptive radiations (e.g. Streelman & Danley, 2003), wing shape in *Blepharoneura* may serve as a template for understanding the complex interplay between courtship, sexual selection, and speciation in host-specific tropical insects.

Acknowledgements

The authors are grateful to the government of Ecuador for permission to carry out this work, which was supported by grants from Cornell College and from the National Science Foundation (NSF-DEB 0330845 to MC, NSF- DEB 0446758 and 0535825 to DCA). The authors are grateful for constructive comments from anonymous reviewers.

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Accepted 4 June 2008