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SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Aedeagal Divergence in Sympatric Populations of Two Sibling Species of Cactophilic *Drosophila* (Diptera, Drosophilidae): Evidence of Character Displacement?

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Abstract

Aedeagal morphology of two sibling cactophilic species, *Drosophila buzzatii* Patterson & Wheeler and *Drosophila koepferae* Fontdevila & Wasserman, was analyzed in nine allopatric and three sympatric locations throughout South America. Morphological differences were detected for both aedeagus size and shape between sympatric and allopatric populations *of D. buzzatii*, despite the significant variability within both groups. Populations of *D. buzzatii* sympatric with *D. koepferae* displayed smaller aedeagus than the allopatric ones as well as more differentiated aedeagus shape. The shape differences were non-allometric and mainly consisted in a change of curvature of the dorsal margin of the aedeagus being more pronounced in males from populations sympatric with *D. koepferae*. It is concluded that aedeagal morphology presented some degree of character displacement in both size and shape in populations of *D. buzzatii* in sympatry with *D. koepferae*. These results might suggest the existence of mechanisms of interspecific recognition and hybridization prevention between these species that include the morphology of the male genitalia.

Introduction

The *Drosophila buzzatii* cluster (*D. repleta* group) comprises at least seven cactophilic species from South America. Male genital morphology is considered the main diagnostic morphological character to species recognition in the cluster (Vilela 1983). The *Drosophila buzzatii* cluster is an excellent material for ecological and speciation studies, due to its morphological and cytological polymorphism and polytypism (Machado *et al* 2002).

Drosophila buzzatii Patterson & Wheeler and *Drosophila koepferae* Fontdevila & Wasserman (Diptera: Drosophilidae), a pair of sibling species of this cluster nearly identical in their external morphology, are reproductively isolated by partial ecological isolation (Fanara *et al* 1999), sexual isolation and postmating barriers (Naveira & Fontdevila 1986). These species present high levels of divergence according to estimations of Nei's genetic distance (Sánchez 1986) or nucleotide divergence (Gómez & Hasson 2003, Piccinali *et al* 2004). However, this isolation could be incomplete. In laboratory, males of *D. buzzatii* can inseminate *D. koepferae* females and female hybrid offspring can be successfully backcrossed with *D. buzzatii* males (Marín & Fontdevila 1998). Furthermore, recent population genetic studies have provided indirect evidence of past or recent gene flow between these species (Gómez & Hasson 2003, Piccinali *et al* 2004), which are undistinguishable by their external morphology, but males present both contrasting aedeagal morphologies and patterns of variation (Soto *et al* 2007). Interspecific hybridization studies in laboratory conditions showed that hybrid genital morphology was not intermediate between parental species, and the morphological resemblance to parental strains was cross-dependent (Soto *et al* 2007). These results suggest a complex genetic architecture, involving genetic factors with major effects (Soto *et al* 2007).

Although studies suggest that hybridization has been of some significance (Gómez & Hasson 2003), the role that it has played in the evolution of *D. buzzatii* and *D. koepferae* remains unclear. Given that aedeagal divergence is the only traceable morphological trait that accompanied the species evolution in the cluster (Manfrin & Sene 2006), and the studies suggesting the possibility of genetic flow between these species as a potential source of intraspecific variation, we wonder how is aedeagal morphological differentiation in regions where *D. buzzatii* coexists and shares resources with *D. koepferae*, its sister species, with respect to allopatric populations. In other words, does the presence of *D. koepferae* affect the evolution of the aedeagus of *D. buzzatii*? If hybridization is a relatively common and recurrent phenomenon, then sympatric populations of *D. buzzatii* should be morphologically less distinct to *D. koepferae* than conspecifics from other allopatric areas where *D. buzzatii* does not coexist with its sibling. If, on the contrary, contact between these species triggered evasion mechanisms of hybridization, and the aedeagus participates in the recognition between species as documented in other species of *Drosophila* (Jagadeeshan & Singh 2006), we would expect an acceleration in the rate of morphological evolution as a result of character displacement (Coyne & Orr 2004) that would cause sympatric populations of *D. buzzatii* to be morphologically more differentiated than allopatric ones.

In this work, I present the geometric quantification of the aedeagus morphology of males from different natural populations of *D. buzzatii,* both sympatric and allopatric with respect to populations of *D. koepferae,* and determine the degree of differentiation among them. The working hypothesis is that populations of *D. buzzatii* in sympatry with *D. koepferae* will present a higher degree of morphological divergence in respect to its sibling than allopatric populations. This divergence might involve one or both components of morphology: the size and/or shape of the aedeagus.

Material and Methods

The sites of fly collection spanned nearly the entire southern portion of the distribution of *D. buzzatii* and *D. koepferae* in Argentina (Fig 1). Flies from 12 natural populations were collected by means of net sweeping over yeast-banana baits and preserved in 70 % ethanol. In the laboratory, males were dissected, their aedeagi removed and mounted on microscope slides, flattened with a cover slip and photographed with a digital camera mounted on a microscope at 400× magnification. Males of *D. koepferae* were captured in populations co-occurring with *D. buzzatii* (Vipos, Valle Fértil and Suyuque; Fig 1), but they were never found in allopatry. A total of 280 males (230 *D. buzzatii* and 50 *D. koepferae*) were analyzed.

The aedeagus is a flat chitinous organ that can be effectively described in shape and size in two dimensions when flattened. I decided to employ an approach of morphological quantification based on elliptic Fourier descriptors (EFDs; Kuhl & Giardina 1982). As described in previous studies (Soto 2005, Soto *et al* 2007), outlines from digital images were used to obtain Fourier coefficients for a polynomial function of 30th degree which were computed with SHAPE v1.2 package (Iwata & Ukai 2002). The area of the left side of each aedeagus (in pixels) was calculated from the digital images and considered as an estimator of the size of the sclerite. Size, orientation and starting position of the contours were standardized in accordance with the size and alignment of the major axis of the first ellipse, leading to representations of the organs that are only based on internal properties of the outlines (i.e., shape) (see Soto 2005 for details). The variance–covariance matrix of the estimated EFD coefficients was used as input in a principal components analysis yielding PC scores for each specimen that can be considered as reorganized uncorrelated morphological traits representing different aspects of total shape variation (Iwata & Ukai 2002) that were used as shape descriptor variables in subsequent analyses.

During the quantification of the organ's shape, I only considered the aedeagus itself excluding the aedeagal apodeme and the paraphysis (Fig 2). Thus, the studied contour was simplified by taking into account only the portion of the organ effectively involved in the penetration of female genitalia. Exploratory analyses applying this morphometric technique showed that it is repeatable and reliable in species discrimination (Soto 2005).

The allometric relationship between aedeagus size and wing length, a trait correlated with overall body size, was also evaluated as the size of adults are known to be affected by the host plant used as larval rearing substrate (Carreira *et al* 2006). The right wing of each captured male was removed and mounted on microscope slides. Wing images were captured using a stereomicroscope (50×) attached to a digital camera connected to a computer. For each wing, we scored the total length (the distance from the intersection of veins II and III to the distal end of vein III in the wing margin; see Carreira *et al* 2006) using TPS DIG (Rohlf 2001).

In order to maximize the description of interspecific differences, a discriminant analysis was performed with species as grouping variable and the PC scores describing each aedeagus as the independent variables. Consequently, a phenetic distance (Mahalanobis squared distance) to the centroid of the other species sample could be assigned to each individual, an estimator of the degree of genital resemblance to its sibling mean morphology.

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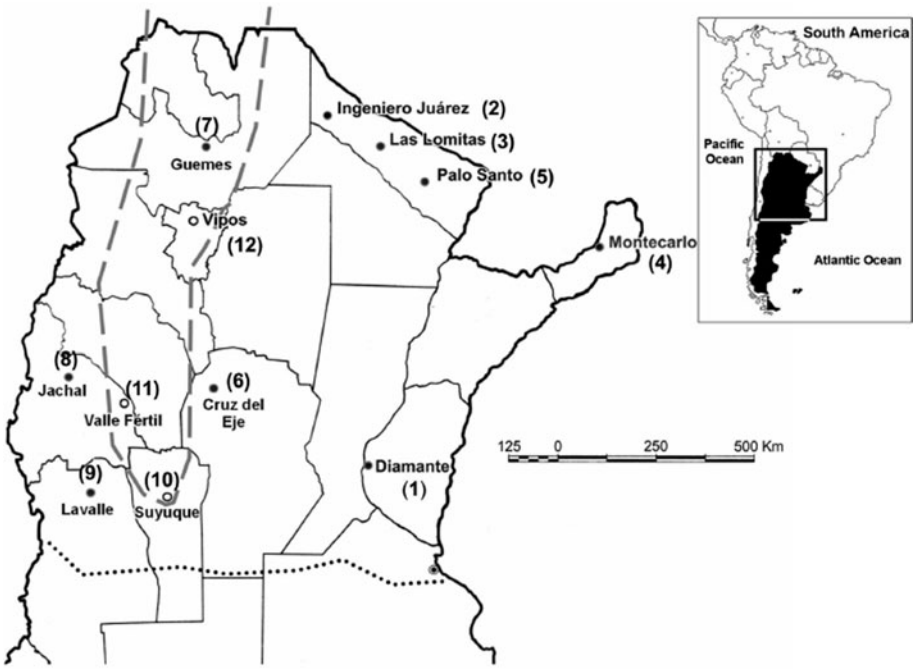

Q4 Fig 1 Natural populations sampled (see also Table 1). *Dotted and dashed lines* marks the southernmost limit of the geographic range of *Drosophila buzzatii* and *Drosophila koepferae*, respectively. Black circles indicate populations of *Drosophila buzzatii* (in allopatry), whereas *white circles* show the location of sympatric populations of *Drosophila buzzatii* and *Drosophila koepferae*. For Güemes sampling, see text.

As stated above, *D. koepferae* was only found in populations coexisting with *D. buzzatti*. Therefore, morphological differences between sympatric and allopatric populations could only be tested in *D. buzzatii*. Analysis of variance (ANOVA) was performed with the phenetic distances as dependent variable and status (sympatry vs. allopatry) and population (random factor nested in status) as independent variables. The same design was also used in an ANOVA with aedeagus size as the dependent variable.

Due to the unequal contribution of sampled populations of *D. buzzatii* to each group (three sympatric vs. nine allopatric populations), I decided to perform a permutation test in order to calculate the probability of obtaining the observed differences between groups by chance alone. Populations were randomly assigned to one of two groups (N=3 or N=9) and the difference between means (small group minus large group) was calculated for both aedeagus size and shape distance. A total of 1,000 permutations were performed in order to estimate a null distribution of the variable. Consequently, I was able to assign to the observed differences between means a probability of being obtained by chance.

Additionally, I performed two separate analyses in SHAPE, one considering only the allopatric populations and the other with the sympatric in order to obtain their respective mean shapes for illustrative and comparative purposes (Fig 3).

Statistical and permutation analyses were performed using the packages Statistica (Statsoft 2007) and PopTools (Hood 2010).

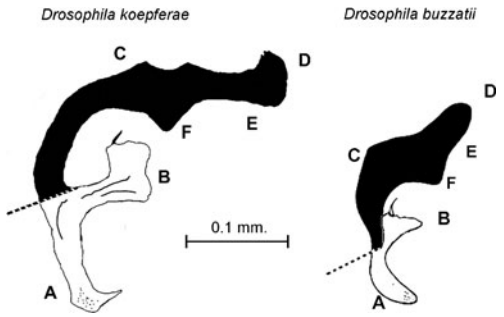

Fig 2 Representation of aedeagus in lateral view of *Drosophila koepferae* and *Drosophila buzzatii.* Black silhouette represents the portion of the organ included in the quantification of size and shape variation. **A** Aedeagal apodeme, **B** paraphysis, **C** dorsal margin, **D** tip, **E** ventral margin, **F** ventral process. Modified from Soto *et al* (2007) and Silva & Sene (1991).

Results

The total number of principal components explaining a significant proportion of shape variation was 12 (accounting for more than 93 % of total shape variance). The cumulative contribution of the first five principal components of the EFDs of the genital outlines accounted for 80.8 % of total shape variance (Table 1).

Table 2 shows the mean aedeagus size and aspect (expressed as a disimilarity index given by the phenetic distance to the mean aedeagus aspect of *D. koepferae*) of each sampled population. On average, *D. koepferae* males captured showed an aedeagus size three times larger than its sibling. There were also marked interspecific differences in aedeagus shape; phenetic distances between species

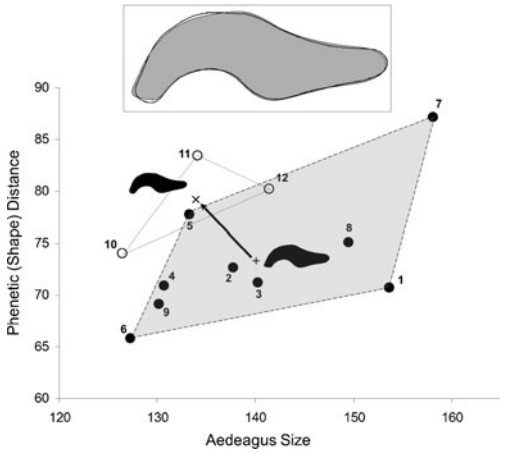

Fig 3 Morphological differences in genitalia between allopatric (# 1–9; *black circles*) and sympatric (# 10–12; *white circles*) populations of *Drosophila buzzatii* ordered by mean aedeagus size (*X* axis, in pixel units) and mean phenetic distance to *Drosophila koepferae* mean shape (*Y* axis, phenetic distances). Population numbers as in Table 1. The *arrow* indicates the displacement from mean allopatric values to the mean sympatric values of both characters which are depicted as *black silhouettes* (mean aedeagus size from sympatric populations is also 7 % smaller). *Inner box* illustrates the shape differences between mean aedeagus shape of allopatric (*grey silhouette*) and sympatric (*white silhouette*) populations without considering size differences.

were approximately 7.5 times larger than that among populations within species.

Morphological differences were detected for both size and shape between sympatric and allopatric populations of *D. buzzatii* (significant "Status" effect, Table 3) despite the significant variability among populations within both groups. Populations of *D. buzzatti* sympatric with *D. koepferae* displayed smaller aedeagus than allopatric ones (134.0 and 140.9 mean aedeagus size, respectively). Additionally, the shape of the former was more differentiated with respect to *D. koepferae* than the latter, as evidenced by the larger phenetic scores in average (79.2 and 73.4 mean phenetic distances in sympatry and allopatry, respectively; Fig 3). The shape differences mainly consisted in the degree of curvature of the posterior portion of the aedeagus being more pronounced in males from sympatric populations (Fig 3).

A possible confounding effect is the possibility that the observed differences in shape were due to size changes (allometric effect). In other words, if the effect is allometric, then the response observed for both traits is in fact only one: a change of aedeagus size. In order to evaluate this effect, I calculated the degree of correlation between the size of aedeagi and their shape. The differences in shape were independent of size changes. The allometric effect on shape differences between allopatric and sympatric populations was negligible, the correlation between aedeagus size and phenetic distance was not significant (Pearson correlation test; r=0.06, P=0.31).

Size of aedeagus and wing length were not significantly correlated in *D. koepferae* (r=0.13, P=0.07), whereas in *D. buzzatii*, a significant allometric relationship (r=0.32, P< 0.001) was detected. Furthermore, aedeagus size and wing length varied isometrically in *D. buzzatii* as suggested by a coefficient of allometry not significantly different from 1

Q2 Table 1 Percent of the original shape variation explained by the principal components (PCs).

PCs	Eigenvalue	Proportion of explained variance (%)	Cumulative (%)
PC 1	3.74E-03	37.08	37.08
PC 2	2.40E-03	23.75	60.83
PC 3	9.22E-04	9.14	69.96
PC 4	6.68E-04	6.62	76.58
PC 5	4.22E-04	4.18	80.76
PC 6	3.66E-04	3.63	84.39
PC 7	2.37E-04	2.35	86.74
PC 8	2.01E-04	1.99	88.73
PC 9	1.69E-04	1.68	90.41
PC 10	1.36E-04	1.35	91.76
PC 11	9.65E-05	0.96	92.72
PC 12	8.97E-05	0.89	93.61

These PCs were subsequently used as shape variables to determine the phenetic distances among groups (see text).

Table 2 Mean aedeagus size (in pixels) and mean phenetic distance to *Drosophila koepferae* mean shape of all sampled populations (standard errors in parentheses).

Species	#	Population	*N*	Genital size	Phenetic distance to *D. koepferae* mean shape
D. buzzatii	1	Diamante	11	153.67 (5.63)	70.64 (4.93)
	2	Ing. Juarez	39	137.73 (2.99)	72.61 (2.62)
	3	Las Lomitas	35	140.26 (3.15)	71.21 (2.77)
	4	Montecarlo	11	130.68 (5.63)	70.93 (4.93)
	5	Palo Santo	35	134.46 (2.51)	74.52 (2.05)
	6	Cruz del Eje	18	127.23 (4.40)	65.80 (3.86)
	7	Güemes	7	158.16 (7.05)	87.15 (6.18)
	8	Jachal	4	149.52 (13.19)	75.09 (11.57)
	9	Lavalle	8	130.20 (9.33)	69.11 (8.18)
	10	Suyuque[a]	11	126.48 (5.63)	74.02 (4.93)
	11	Valle Fértil[a]	16	141.36 (4.66)	80.17 (4.09)
	12	Vipos[a]	35	134.09 (3.15)	83.47 (2.77)
D. koepferae	a	Suyuque	23	482. 85 (4.22)	11.34 (3.41)
	b	Valle Fértil	12	337.26 (5.37)	8.89 (4.72)
	c	Vipos	15	385.13 (4.74)	9.68 (4.22)

[a] Those populations of *Drosophila buzzatii* in sympatry with *Drosophila koepferae.*

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t3.1 Table 3 ANOVA testing for
t3.2 aedeagus size and shape (phenetic distances) variation among
t3.3 populations of *Drosophila buzzatii* and between allopatric and sympatric groups.

Trait	Sources of variation		*df*	MS	*F*	
Aedeagus size	Status	Fixed	2	10,990.91	1241.10**	
	Population (status)	Random	10	11.11	3.19**	t3.4
	Error		212	3.48		t3.5
Aedeagusl shape (Phenetic distance)	Status	Fixed	2	334,908.3	858.13**	
	Population (status)	Random	10	419.0	1.30	t3.7
	Error		212	321.9		t3.8

t3.6 *df* degrees of freedom, *MS* mean squares

**$P<0.01$.

(slope value of linear adjusted function=0.82; 95 % confidence interval values from 0.42 to 1.23).

By means of resampling, null distributions were estimated for size and shape differences between two groups of populations. The probability of obtaining the observed differences in aedeagus size by chance was below the 5 % conventional limit ($P=0.04$) as was also the assigned probability for the observed differences in phenetic distance ($P=0.032$). Considering both responses as independent, we had a combined probability lower than 0.01 ($P=0.0013$) for the observed character displacement in aedeagal morphology of *D. buzzatii*.

Another important confounding factor is the high morphological variability within populations. The original shape variables were used in a discriminant analysis to assess to what degree the data dispersion around the means overlaps. Thus, I tested whether the aedeagal morphology could function as predictor of the membership of each individual to a population allopatric or sympatric with *D. koepferae.* The analysis showed a posterior probability of correct reassignment of 81 %. This result indicated that despite the significant overlapping between groups, there is enough morphological differentiation to identify individuals with probabilities higher than expected by chance alone.

Discussion

The evolutionary relevance of natural occurring hybridization depends critically on the fitness of hybrids, an issue that has been extensively discussed elsewhere (Burke & Arnold 2001, Coyne & Orr 2004 and references therein). In *Drosophila* species, interspecific hybridization has been considered a phenomenon unlikely to be observed in nature (Coyne & Orr 2004). However, recent molecular population genetic surveys in the *D. buzzatii* cluster yielded evidence that raised the possibility of interspecific gene flow (either ancient or recent), which may be assumed as an additional source of within species variation. Studies of nucleotide variation in nuclear loci have shown the presence of shared single nucleotide polymorphisms between *D. buzzatii* and *D. koepferae* (Gómez & Hasson 2003, Piccinali *et al* 2004). In fact, studies of mtDNA indicate that interspecific hybridization may have played a pervasive and significant evolutionary role in the cluster as other species pairs showed evidence of interspecific allele flow, as it was reported for *Drosophila serido* Vilela & Sene and *Drosophila antonietae* Tidon-Sklorz & Sene 2001, and between the latter and *Drosophila gouveai* Tidon-Sklorz & Sene 2001 (Manfrin *et al* 2001).

Several isolation barriers are well established between *D. buzzatii* and *D. koepferae*. Gene flow between these species is restricted by sexual (Marín *et al* 1993) and partial habitat isolation (Fanara *et al* 1999). Machado *et al* (2002) studied the reproductive isolation within the *D. buzzatii* cluster and observed absence of copula (or interrupted copula) as pre-zygotic reproductive isolation in several attempted crosses. Furthermore, they observed that pre-zygotic isolation was stronger among sympatric strains/species.

Nevertheless, besides these barriers, hybrids are produced in laboratory conditions, even though in low numbers: hybrid zygotes that reach adulthood represent only a small proportion of the fertilized eggs laid by females, indicating that egg viability of hybrids is extremely low (Naveira & Fontdevila 1986, Soto *et al* 2008). This may reflect a high degree of incompatibility between parental genomes which limits development beyond the initial embryonic stages. On the other hand, the hybrid offspring that reach adulthood consist on fertile females and sterile males. Hybrid females can be backcrossed with *D. buzzatii* males (backcross in the direction of *D. koepferae* males is extremely unlikely), and male fertility can be restored after a few generations of backcrosses (Naveira & Fontdevila 1986). Fitness of this fraction of surviving hybrids is not lower than the parental species when host-related developmental time and viability is considered (Soto *et al* 2008).

In nature, the possibility of hybridization exists; these species spend their larval period submerged and feeding on cacti rots, whereas adults feed on the same decaying tissue where they also mate and lay their eggs (Santos *et al* 1988, Fanara *et al* 1999). Despite a certain degree of host specificity, flies from both species are usually seen feeding on the same plant and emerging as adults from the same substrates in sympatric populations (Fanara *et al* 1999).

Thus, given the intrinsic reduction of hybrid fitness due to meiotic irregularities between *D. buzzatii* and *D. koepferae*, a selective pressure to avoid interspecific mating in sympatry would be expected.

Variations in aedeagus provide an opportunity for cryptic female choice and species discrimination. Copulatory mechanics differ between very closely related *Drosophila* species (Jagadeeshan & Singh 2006). Both *D. buzzatii* and *D. koepferae* present genetic variability for aedeagus morphology (Soto *et al* 2007). Thus, if aedeagus morphology is involved in mechanisms that prevent interspecific mating, a differential morphological evolution in sympatric populations as compared with allopatric ones could be observed.

In this study, we observed that (a) different values of both aedeagus size and shape of sympatric versus allopatric populations of *D. buzzatii* and (b) interspecific differences in sympatry with *D. koepferae* were larger than those in allopatry.

The detected variation between populations of *D. buzzatii* that are sympatric and allopatric with *D. koepferae* may be due to some environmental factors. Since *D. koepferae* is restricted to a narrower geographic range, probably at higher altitudes, the sympatric populations of *D. buzzatii* would also be living under different environmental conditions from those of its allopatric populations. Thus, the posibility that the detected morphological differences may have been caused by or be byproducts of adaptations to such different environmental conditions cannot be entirely ruled out with the present data. However, it is worth noting that shape differences between allopatric and sympatric populations were non-allometric, preventing any significant effect of any environmental factor affecting body size.

Another possibility is the existence of a phylogenetic constraint. If there is phylogenetic (genetic) differentiation between the sympatric and the allopatric populations of *D. buzzatii*, i.e., the sympatric ones form a clade, the aedeagal morphological characteristics seen in the sympatric populations can be interpreted as resulting from the phylogenetic or historical effect. However, the most extensive survey up to date of genetic structuration on *D. buzzatii* found that genetic differentiation among populations within and among regions for total, nonsynonymous, synonymous, and silent variation was not significant either in Argentinian or Australian populations (Piccinali *et al* 2007). This absence of population structure in *D. buzzatii* was interpreted as evidence of extensive gene flow among populations and/or recent divergence from an ancestral stock and discarding the scenario of a single monophyletic origin of sympatric populations (Rodríguez *et al* 2000, Piccinali *et al* 2007).

Remarkably, the population of *D. buzzatii* with the most dissimilar aedeagal morphology with respect to *D. koepferae* was an allopatric population (Güemes). However, despite failure to find any males of *D. koepferae*, this population is within the range of this species distribution. One possibility is that we are actually dealing with a population with a recently acquired allopatric status due to local extinction of its sibling species. In fact, due to the patchy distribution of both species (associated with the discontinuous distribution of their cactus hosts), local extinctions and subsequent recolonization are not uncommon (Moraes & Sene 2002, Piccinali *et al* 2004). However, regardless of the values observed in the individuals from this locality (conservatively included as allopatric in the analyses), general differences between sympatric and allopatric populations could still be detected.

In summary, based on the present evidence, character displacement seems to be the most plausible explanation for aedeagal divergence in both size and shape in populations of *D. buzzatii* sympatric with *D. kopeferae*. These results might be suggesting the existence of mechanisms of interspecific recognition and prevention of hybridization that include the male genitalic morphology although further studies are needed in order to confirm the present results.

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Q1. Please check Affiliation if captured correctly.
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