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RESEARCH ARTICLE

Differential Rates of Male Genital Evolution in Sibling Species of *Drosophila*

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Abstract

Genital morphology in animals with internal fertilization is considered to be among the fastest evolving traits. Sexual selection is often proposed as the main driver of genital diversification but the exact selection mechanisms involved are usually unclear. In addition, the mechanisms operating may differ even between pairs of sibling species. We investigated patterns of male genital variation within and between natural populations of the cactophilic fly *Drosophila koepferae* ranging its entire geographic distribution and compared them with those previously observed in its sibling species, *D. buzzatii*. Using both mtDNA and nDNA markers we found that genital shape variation in *D. koepferae* is more restricted than expected for neutral evolution, suggesting the predominance of stabilizing selection. We also detected dissimilar patterns of divergence between populations of *D. koepferae* that were allopatric and sympatric with *D. buzzatii*. The constrained evolution inferred for *D. koepferae*'s genitalia clearly contrasts with the rapid divergence and higher morphological disparity observed in the populations of *D. buzzatii*. Finally, different possible scenarios of male genital evolution in each species and within the radiation of *D. buzzatii* cluster are discussed.

Keywords Aedeagus · Morphological disparity · Morphological evolution · Sexual selection

Introduction

In animals with internal fertilization, genital evolution is a rapid phenomenon thought to be mainly driven by selective processes (Eberhard 1985, 2010; Hosken and Stockley 2004). Despite the increasing amount of research on this topic, much of the mechanisms involved remain unclear (Simmons 2013; Brennan and Prum 2015). Recent studies suggest that divergence of genital morphology could be a more complex phenomenon than previously thought and be driven, even antagonistically, by different selective processes in different populations within the same species (Simmons et al. 2009; Rowe and Arnqvist 2012; Simmons 2013; Brennan and Prum 2015; Anderson and Langerhans 2015). These processes could also alternate through time to give rise to different patterns of evolutionary change (McPeek et al. 2008).

Three main groups of hypotheses were proposed to account for the evolution of genital morphology: "Lock-and-Key" (Dufour 1844), Pleiotropy (Mayr 1963) and sexual selection (Eberhard 1993). "Lock-and-Key" considers that male genitalia evolve as a species-specific trait and is constrained to physically fit the female genitalia. This hypothesis predicts both limited phenotypic variation and low levels of genetic variance in genital morphology (Shapiro and Porter 1989; Arnqvist 1997). The Pleiotropy hypothesis assumes that genital traits evolved due to genetic correlations with other evolutionary relevant non-genital traits. Thus, changes in allele frequencies at pleiotropic loci affecting both somatic morphology and genital morphology may

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lead to evolution of genital traits at arbitrary rates and morphological directions. Finally, the sexual selection hypothesis predicts a correlation between variation in male genital morphology and fitness via processes such as sexual conflict, cryptic female choice or male–male competition (Brennan and Prum 2015). All these processes can promote rapid genital divergence under continuous directional selection (Hosken and Stockley 2004).

The genus *Drosophila* includes some species that have been used as model for the study of genital evolution in insects. For instance, the male intromittent organ (aedeagus) is considered the main diagnostic morphological trait for species recognition in the *D. repleta* group (Vilela 1983) and its divergence was studied recently in several species (Soto et al. 2007, 2008a, 2013; Soto 2012; Richmond et al. 2012). *Drosophila koepferae* and *D. buzzatii* are Neotropical cactophilic sibling species members of the *D. buzzatii* cluster. They can breed and feed on the necrotic tissues of several cacti species (Soto et al. 2012) but exhibit a certain degree of niche separation; *D. koepferae* prevails in necrotic pockets of columnar cacti of the genera *Cereus* and *Trichocereus*, whereas *D. buzzatii* is mainly adapted to breed on decaying prickly pears of genus *Opuntia* (Oliveira et al. 2012). Their recent demographic histories are contrasting: while *D. koepferae* is restricted to arid regions of northwestern Argentina and southern Bolivia, its sibling species is also found in central Argentina, Brazil, Paraguay and in the past centuries have reached extended global distribution (Africa, Australia, Mediterranean), following the human-driven dispersal of prickly pears. This pattern is mirrored by their respective population genetics: *D. koepferae* has low frequency or lack of gene flow among structured populations, a feature related to the isolated distribution of its cactus hosts (Piccinali et al. 2004; Lipko 2013). In contrast, *D. buzzatii* is genetically less variable than *D. koepferae* and presents signs of recent demographic expansions in southern South America, showing no significant genetic structuration (Piccinali et al. 2004; Lipko 2013; Soto et al. 2013). Both species can be found in sympatry in most of the distribution range of the former (see below), and are reproductively isolated by partial ecological segregation (Fanara et al. 1999) and post mating barriers (Naveira and Fontdevila 1986; Soto et al. 2007).

Although synmorphic in their general aspect, these species present striking morphological differences in their genitalia. *Drosophila buzzatii* exhibits a characteristically small genitalia (one-third of the size of that of *D. koepferae*) with a distinctive shape (Fig. 1a), whereas *D. koepferae* aedeagal size and shape are more similar to that of the other species of the cluster (Fig. 1a, b). Comparative analyses of aedeagus morphology within the *D. buzzatii* cluster indicate a close relationship of *D. koepferae* with *D. serido* and allied species (Manfrin et al. 2001; Fig. 1b). However, mitochondrial DNA phylogenetic analyses place it as the sister species of *D. buzzatii* (Manfrin et al. 2001; Manfrin and Sene 2006; Oliveira et al. 2012). According to previous studies, *D. buzzatii*'s male genital morphology has rapidly diverged after the recent demographic expansion of the species. In this species, a model of drift-facilitated, either sexual or natural selection has been suggested to account for genital evolution (Soto et al. 2013). Therefore rates of genital evolution may be heterogeneous between sibling species and among branches

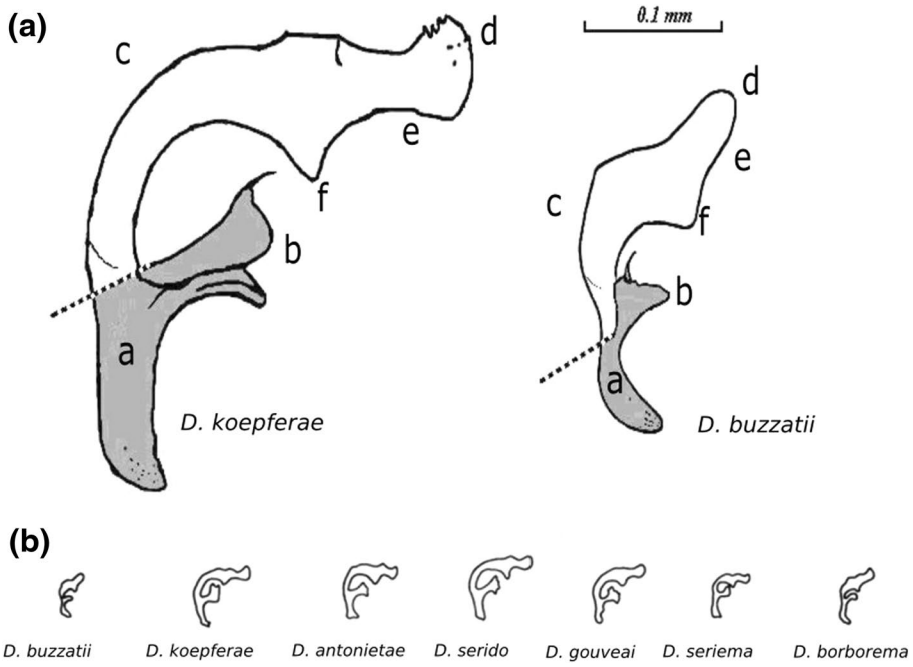

Fig. 1 **a** Lateral view of aedeagus of *Drosophila koepferae* and *D. buzzatii*. Grey areas represent the portion excluded from shape quantification. *a* Aedeagal apodeme, *b* paraphysis, *c* dorsal margin, *d* tip, *e* distal ventral margin, *f* ventral process (modified from Soto et al. 2007); **b** aedeagus of the seven recognized species of the *Drosophila buzzatii* cluster

of the clusters' phylogenetic tree but also differences in the genetic structure behind the development of genitalia could be expected, even between closely related species. Different evolutionary processes involved in the evolution of genital morphology of these species might lead to very dissimilar evolutionary pathways. This is a call for both intra and interspecific comparisons and sets this group of species as an excellent system to study genital evolution.

Here, we performed the first comparative study of genital evolution in this cluster of cactophilic *Drosophila*. We assessed the patterns of intraspecific genital variation in *D. koepferae* and determined whether is compatible with expectations under random drift-gene flow balance or alternatively hypothesis including selective processes. Additionally, we compared these results with those previously reported by Soto et al. (2013) for *D. buzzatii*, while simultaneously studying the degree of genital morphological disparity elicited by each species since their divergence.

Materials and Methods

Flies were collected with baited traps in populations of *D. koepferae* distributed throughout northwestern Argentina and southern Bolivia (Fig. 2). Isofemale lines of each population were maintained in bottles with 30 ml. of standard *Drosophila* instant medium (Carolina Biological Supply Company) and transported to the laboratory. Rearing conditions were held constant at 25 ± 1 °C with a 12:12 h light/dark photoperiod.

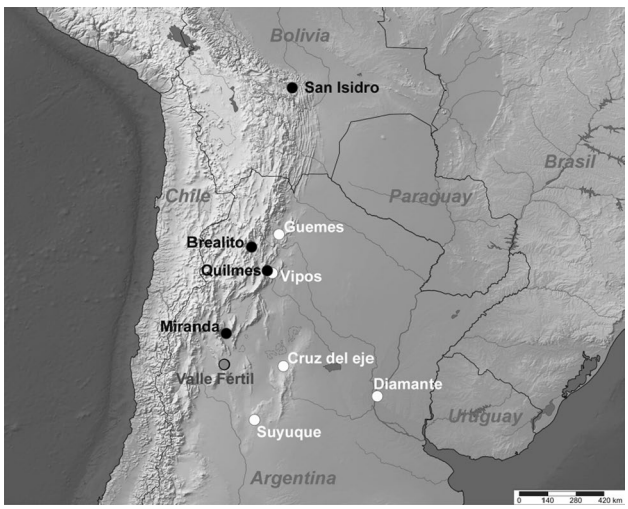

Fig. 2 Natural populations of *D. koepferae* (black dots) and *D. buzzatii* (white dots) sampled. Both species were found in sympatry in Valle Fertil (grey dot)

Morphological Quantification

A total of 59 aedeagi (12 per population, one discarded after mounting) from adult males were dissected, mounted on microscope slides and flattened with cover slips with DPX (Sigma-Aldrich). Slides were photographed at 400× magnification with a camera mounted on a microscope (for more details see Soto et al. 2007). Aedeagal morphology was quantified as an open outline using the discrete cosine transform (DCT; Dommergues et al. 2007), a Fourier-related technique that allows to measure the shape of open outlines and curves by decomposing them into a series of harmonics (sine and cosine functions) and using the Fourier coefficients (two per harmonic) as shape descriptors (Soto et al. 2007). Effects of size, rotation and position were removed by performing Generalized Procrustes Analysis (Gower 1975) on the raw (x, y) coordinates prior to application of DCT. Twenty-five harmonics were enough to capture aedeagal morphological complexity across the entire sample [apodeme and paraphyses were excluded from quantification in both species (Fig. 1)]. DCT was performed in the R environment (R Core Team 2016) using the Momocs package (v. 2.0-6; Bonhomme et al. 2014).

The variance–covariance matrix of the estimated coefficients was used to perform a principal component analysis (PCA). This procedure allowed us to summarize and reduce the dimensionality of the shape information described by the coefficients. The resulting scores of each PC could be considered as reorganized uncorrelated morphological traits representing different aspects of total shape variation (Soto et al. 2007). After applying the Broken-Stick method (Jackson 1993), we retained five PCs (which describes about 90% of the total shape variation) for the subsequent statistical analyses. The original area of each outline was used as a proxy of genital size.

We worked with two sets of PC scores following Soto et al. (2007). The first set was calculated from the matrix of coefficients derived exclusively from outlines of the genitalia of males of *D. koepferae*, improving the assessment of intraspecific variation in aedeagus morphology by avoiding the noise resulting from conspicuous interspecific morphological differences in the estimation of the PCs. The other set included all outlines belonging to both *D. koepferae* and *D. buzzatii*, thus allowing the evaluation of interspecific differences in genital morphological disparity in a common morphospace.

Although a previous study in *D. koepferae* showed no effect of body size on aedeagus size (Soto et al. 2007) we still assessed the degree of correlation between genital and body size (estimated from wing length) and whether interpopulational differences in genitalic size were exclusively allometric (i.e., a direct consequence of body size). Differences among populations of *D. koepferae* in genital size

were evaluated by means of an ANOVA using the aedeagus area (size as in Garnier et al. 2005) as the dependent variable. Assumptions of the model were previously tested. To determine whether the interpopulation variation in shape was significant, a NPMANOVA (Non-parametric MANOVA, also known as PERMANOVA; Anderson 2001) was performed using the retained PC scores as dependent variables (Tabachnick and Fidell 2007). Aedeagal size was included in the model as a covariate in order to assess non-allometric shape variation (Soto et al. 2007). The empirical distribution of pseudo-F values was obtained via 9999 permutations of residuals under the null model of no interpopulational differences (Anderson 2001; McArdle and Anderson 2001). Pairwise NPMANOVAs (with Bonferroni correction) were performed between all pairs of populations as a post-hoc test assessing which pairs presented significant differences. All analyses were performed in the R environment using the vegan package (Oksanen et al. 2017).

Interpopulational Genetic Divergence of *D. Koepferae*

We obtained estimators of the degree of interpopulational genetic differentiation from previous population genetics studies addressing the same populations (Lipko 2013). *F*ST distances (Wright 1951) were calculated using both microsatellite loci and sequence variation in the mitochondrial *cytochrome oxidase* subunit I (COI). The former marker is widely used for testing neutral genetic divergence (Leinonen et al. 2013). Additionally, we decided to include pairwise FST_{COI} in order to compare our results with those previously obtained by Soto et al. (2013) for *D. buzzatii*. Full details on DNA extraction and amplification protocols are described elsewhere (Lipko 2013). Briefly, nine highly polymorphic microsatellites were amplified for 48 individuals from four of five populations included in the morphological assays (the San Isidro population was not included; Lipko 2013). Regarding COI sequence, a segment of 552 pb was amplified for 72 specimens belonging to all the five populations (Lipko 2013). *F*ST statistics (Wright 1951) and pairwise *F*ST distances between populations were calculated for both markers using Arlequin v 3.15 (Excoffier et al. 2009). Different tests were performed in order to assess if both the microsatellites and the COI gene sequence variation of *D. koepferae*'s populations used herein matched the values expected under neutral evolution [Fs (Fu 1997), Tajima Dt (Tajima 1989) and Pairwise Mismatch Distribution (Rogers and Harpending 1992); See Lipko 2013 for full details on neutral tests]. Sequence variation in both markers complied with the expectations under neutral evolution.

Genetic Versus Morphological Divergence in *D. Koepferae*

Mantel tests were performed in order to assess correlations among genetic, phenetic and geographic distances. Pairwise *F*ST distances were transformed to $FST/(1-FST)$ following Rousset (1997). The phenetic distances (squared Mahalanobis distances) were used as multivariate measures of morphological distances (Hankison and Ptacek 2008; Wojcieszek and Simmons 2012). Linear geographic distances among populations were obtained using Google Earth Pro v 7.3.0.3832. The analyses were performed in the R environment using the vegan package.

Pairwise *P*ST were calculated among all populations of *D. koepferae* using data of aedeagus size and shape (first five PCs) separately. Comparing these morphological distances matrices with pairwise *F*ST (not transformed) of COI and microsatellites allowed us to assess whether interpopulational morphological divergence have been driven by selection (Leinonen et al. 2006; Wojcieszek and Simmons 2013; Soto et al. 2013). *P*ST values were calculated using the formula:

$$\mathrm{PST} = \frac{\sigma_B^2}{\left(\sigma_B^2 + 2\sigma_W^2\right)} \quad (1)$$

where $\sigma_B{}^2$ and $\sigma_W{}^2$ are "between" and "within" (or inter- and intra-) population variance, respectively (Raeymaekers et al. 2007). Variance components for *P*ST estimation were obtained through one-way ANOVAs for each trait as the dependent variable and pairwise population combinations as the independent variable, using Statistica version 7 (Stasoft Inc., USA).

In order to assess whether *F*ST was significantly different from *P*ST values (i.e., whether the observed morphological variation is in the level expected under a drift/gene-flow balance) the bootstrapped means and their 95% confidence limits were calculated for *F*ST mean, each *P*ST mean and aedeagus size. The hypothesis of neutral evolution (i.e., drift/gene-flow balance) may be rejected when *P*ST scores are significantly different from *F*ST. A *P*ST larger than *F*ST suggests rapid morphological divergence among populations, and may reflect a directional process like sexual selection and/or sexual conflict (House and Simmons 2003; Hosken and Stockley 2004). A *P*ST smaller than *F*ST may be interpreted as evidence of stabilizing selection driving the morphological genital evolution within populations (Soto et al. 2013).

Adaptation was inferred evaluating additive genetic divergence in quantitative traits across populations (*Q*ST; Spitze 1993) and whether it exceeds the neutral expectation (based on differentiation of neutral alleles across

these populations; *F*ST). Although *Q*ST estimation is not always possible (Leinonen et al. 2013) it can be approximated by *P*ST calculation. The disadvantage of this approach is that *P*ST includes the non-additive genetic variance resulting from environmental factors and genotype by environment interactions. If this is not taken into account, *P*ST may overestimate the amount of additive genetic variance (Pujol et al. 2008). One would expect that, in the wild, environmental effects play a larger role in determining phenotypic variance across populations than within populations. Hence, the proportion of total variance due to additive genetic effects across populations (c) would be equal to the heritability of the trait (h^2). Fulfillment of this conservative assumption would improve *P*ST as good proxy of *Q*ST (Brommer 2011). We assessed if our data violated this assumption by observing the lower critical c/h^2 ratio where *P*ST becomes equal to the upper confidence limit of *F*ST. The lower the critical c/h^2 ratio is for a statistically significant difference between *P*ST and the neutral expectation, the more robust is the inference of selection (Brommer 2011). Finally, we assessed the existence of correlation between pairwise *F*ST (not transformed) and *P*ST, as well as between the two sources of neutral genetic data (i.e., FST_{COI} and FST_{MS}) through simple Mantel tests.

Interspecific Differences in Morphological Disparity of Male Genitalia

As previously stated, a PCA was performed on shape descriptors of the genitalia of specimens belonging to both *D. koepferae* and *D. buzzatii*, in order to account for the interspecific morphological variation. Aedeagi from *D. buzzatii* were obtained from the photographic database used to assess intraspecific patterns of genital variation in a previous study (Soto et al. 2013), and were originally obtained following the same protocol of dissection and photography described for *D. koepferae*. Although *D. buzzatii* is widespread across South America, we decided to analyze a number of populations and a geographic range comparable to those of *D. koepferae*, in order to avoid biases in the results due to inclusion of extremely isolated and/or distant populations (Fig. 2). Therefore, 98 specimens of *D. buzzatii* representing six populations (Fig. 2) were included in subsequent analyses.

The morphological disparity (Foote 1993, 1997) attained by each species was estimated as the sum of the variances of the PC scores of each species. Statistical significance of the observed difference between the so obtained disparities was assessed through a permutation test (9999 random permutations). These analyses were performed in the R environment (R Core Team 2016).

Results

Genital Divergence Among Populations of *D. koepferae*

Body size (estimated through wing length) was not correlated with genital size (Pearson correlation $r = -0.17$, $p = 0.07$) in our sample, ruling out its influence as confounding factor via allometric effects. Mean genital size significantly differed among populations (Table 1a). In contrast, statistical differences in intraspecific genital shape were not found (Table 1b). The retained PCs accounting for genital shape variation are depicted in Fig. 3. Variation along PC1 (37.26% of the total shape variation) comprised mostly morphological changes of the ventral process and aedeagal tip, with positive values associated with an expanded ventral process and an accentuated tip. PC2 (20.72% of the total shape variation) explained variation related with the posterior development of the ventral process and variation in general thickness of the aedeagus, with thicker phenotypes located towards positive values. PC3 (16.77% of the total shape variation) accounted for variation in curvature of the posterior region of the dorsal margin, being the aedeagi with more pronounced curves and rounded dorsal margins located towards the negative values of this axis (positive values represent specimens with squared dorsal margins). PC4 (5.87%

Table 1 Analyses of variance of male genital morphology among populations of *D. koepferae*: (a) ANOVA and (b) NPMANOVA

(a) Size	d*f*	MS	F
Population	4	1.611	2.022*
Error	58	0.792	
(b) Shape	d*f*	MS	R2	F
Populations	4	0.994	0.111	1.944
CS	1	2.152	0.063	4.198*
Populations: CS	4	0.483	0.054	0.943

*$p < 0.05$ after Bonferroni correction

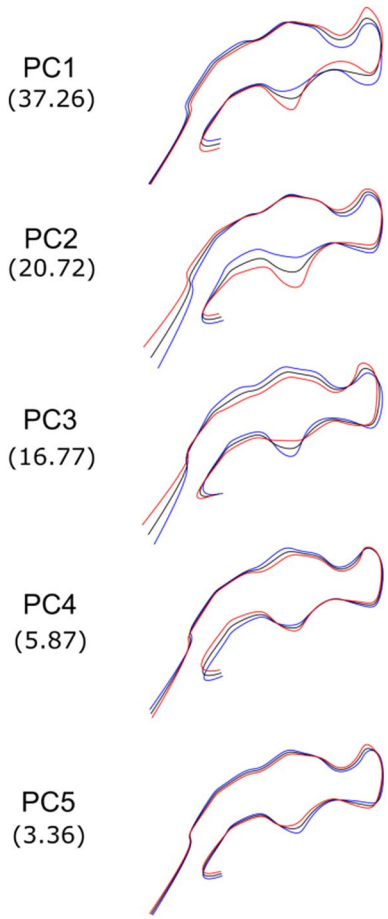

Fig. 3 Shape variation of *D. koepferae*'s aedeagus accounted by the first five principal components (PC) and accounted percentage of original variation (in parenthesis). Black, red and blue lines represent mean shape, mean plus 2 SD and mean minus 2 SD respectively. (Color figure online)

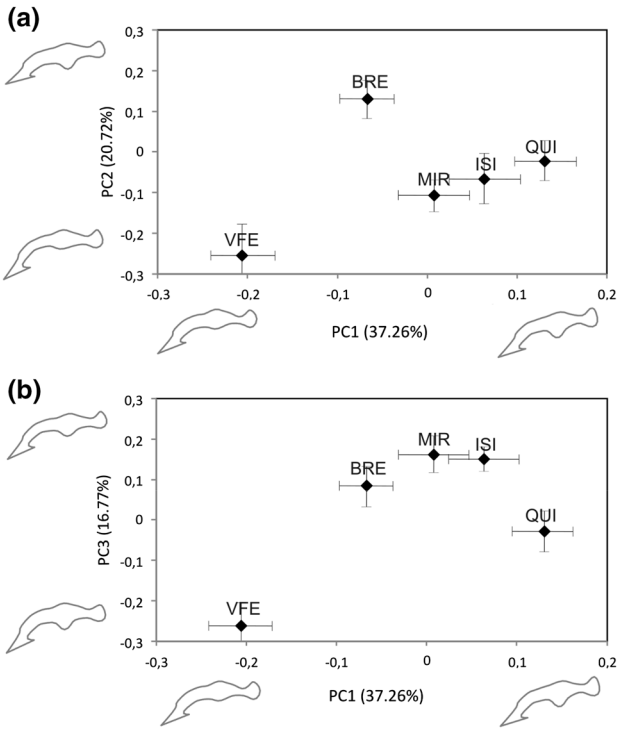

Fig. 4 Plot of mean genital shape scores for each *D. koepferae*'s population. The first three principal components accounting for shape variation and the percentage of variance explained for each one of them (between parentheses) are depicted: **a** PC1 versus PC2 and **b** PC1 versus PC3. Outlines of aedeagi by each axis represent genital shape variation accounted by each principal component. *VFE* Valle Fértil, *BRE* Brealito, *MIR* Miranda, *ISI* San Isidro, *QUI* Quilmes; see map in Fig. 2

of the total shape variation) explained relative changes in size of the proximal portion of the aedeagus and PC5 (3.36% of the total shape variation) accounted for subtle variation in shape of the anterior margin of the ventral process. Figure 4 illustrates the intraspecific morphospace positioning each *D. koepferae*'s population according to their mean genital shape in the first three principal components. Valle Fértil (VFE) stood out as the most dissimilar population in all shape dimensions with a slimmer recurved aedeagus with an underdeveloped ventral process (Fig. 4; Table 2b).

Genetic Versus Phenetic Divergence Among Populations

Analyses performed with *F*ST pairwise distances yielded the same results with both COI and microsatellites. Thus, for the sake of synthesis, we present the *P*ST versus *F*ST comparisons for both molecular markers but the remaining results obtained from FST_{COI} are provided in Supplementary Information. Phenetic, genetic and geographic distances were uncorrelated between each other (r values < 0.24; $p > 0.05$ in all cases). Matrices of pairwise *F*ST (not transformed) obtained for both microsatellites and COI were not correlated with each other ($r = 0.821$; $p = 0.0833$). The five *P*ST of shape and the *P*ST of size were not correlated with pairwise *F*ST (not transformed) (all r values < 0.7; all $p > 0.05$) (Fig. 5). Table 2 shows the distances (geographic, phenetic, morphological and genetic for both molecular markers) calculated for the sampled populations.

Mean PST_{size} and PST_{PC2} values were undifferentiated from mean FST_{COI} (Table 3a) preventing the rejection of neutral evolution as hypothesis for explaining divergence in these aspects of morphology. In contrast, PST_{PC1} values were significantly lower than FST_{COI}, a pattern consistent with the predictions of stabilizing selection. On the other hand, PST_{PC3} to PST_{PC5} means (dimensions accounting for 26% of total shape variation) were significantly larger than mean FST_{COI} (Table 3) suggesting that non neutral evolution but a faster process would explain the divergence pattern in these shape dimensions.

Table 2 (a) Geographic distances (km, below diagonal), genetic *F*ST obtained from Microsatellites and *F*ST obtained from COI (above diagonal). All *F*ST values were taken from Lipko (2013); (b) morphological distances *P*STpc1 and *P*STpc2 (above diagonal) and phenetic distances (squared Mahalanobis distances; below diagonal)

	Brealito	Quilmes	Valle Fértil	San Isidro	Miranda
(a)					
Brealito	_	0.575/0.071	0.723/0.264	NA/0.297	0.620/0.049
Quilmes	160	_	0.560/0.150	NA/0.630	0.747/0.247
Valle Fértil	632	480	_	NA/0.843	0.815/0.509
San Isidro	838	987	1466	_	NA/0.113
Miranda	469	331	179	1306	_
(b)					
Brealito	_	0.458/0.369	0.233/0.662	0.151/0.312	0.071/0.587
Quilmes	0.057	_	0.670/0.496	0.046/0.026	0.199/0.174
Valle Fértil	0.301	0.212	_	0.356/0.201	0.336/0.243
San Isidro	0.105	0.090	0.246	_	0.020/0.025
Miranda	0.014	0.065	0.270	0.107	_

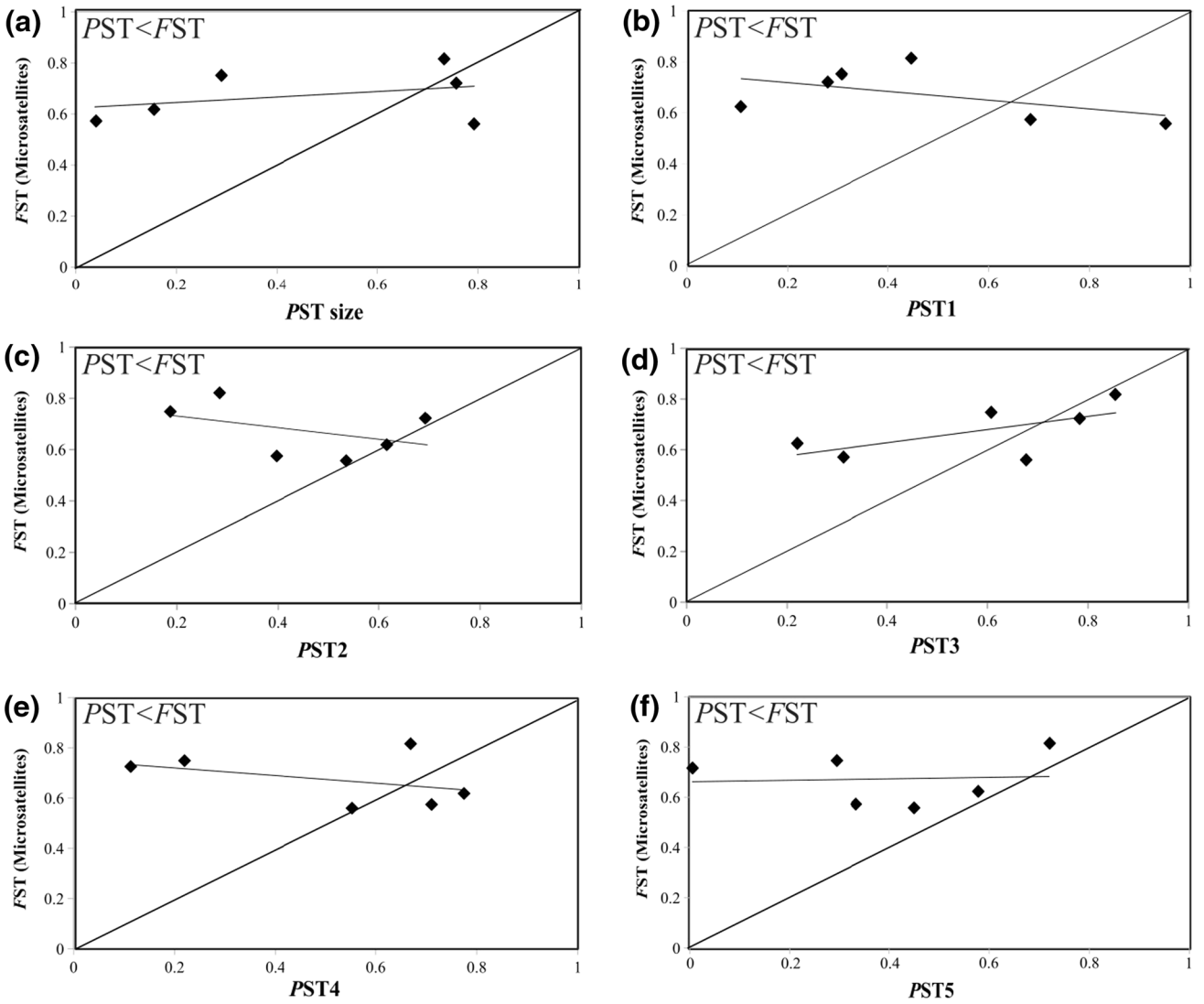

Fig. 5 Plots of phenotypic (*P*ST) differentiation compared to putative neutral genetic differentiation (*F*ST) among populations for the male genital size (**a**) and first five shape variables (**b**–**f**). *F*ST showed are those obtained from microsatellites (FST_{MS}). Lines represent theoretical *P*ST = *F*ST as expected by neutral evolution

Table 3 Means and upper and lower 95% confidence intervals following bootstrapping for pairwaise *F*ST and *P*ST for populations of *D. koepferae*. **a** Pairwise *F*ST obtained from COI from five populations; **b** pairwise *F*ST obtained from nine microsatellites from four populations (i.e., the same COI's populations without San Isidro)

	Mean	Lower 95% confidence limit	Upper 95% confidence limit		Critical c/h^2 value
(a)					
*F*ST (COI)	0.319	0.303	0.335	–	–
*P*ST size	0.343	0.323	0.363	*P*ST = *F*ST	1.054
*P*ST pc1	0.265	0.253	0.278	*P*ST < *F*ST	1.490
*P*ST pc2	0.306	0.293	0.320	*P*ST = *F*ST	1.216
*P*ST pc3	0.482	0.464	0.501	*P*ST > *F*ST	0.583
*P*ST pc4	0.436	0.419	0.452	*P*ST > *F*ST	0.697
*P*ST pc5	0.478	0.461	0.494	*P*ST > *F*ST	0.589
(b)					
*F*ST (Microsatellites)	0.669	0.663	0.675	–	–
*P*ST size	0.464	0.445	0.484	*P*ST < *F*ST	2.594
*P*ST pc1	0.325	0.312	0.338	*P*ST < *F*ST	4.573
*P*ST pc2	0.452	0.440	0.464	*P*ST < *F*ST	2.641
*P*ST pc3	0.569	0.554	0.584	*P*ST < *F*ST	1.670
*P*ST pc4	0.508	0.492	0.524	*P*ST < *F*ST	2.148
*P*ST pc5	0.404	0.390	0.419	*P*ST < *F*ST	3.255

The critical c/h^2 ratio calculated following Brommer (2011) are provided

In contrast to the results obtained using mtDNA, all *P*ST of shape (accounting for 84% of total shape variation) and PST_{size} were significantly lower than mean pairwise *F*ST obtained from microsatellites (FST_{MS}; Table 3b). According to these results all shape dimensions and size might have been under stabilizing selection, at least for the populations with microsatellite data available.

Overall, neither FST_{COI} nor FST_{MS} pairwise comparisons with *P*ST estimates fell upon the line of neutrality (Figs. 5 and Supplementary Information S1), suggesting that genetic drift did not fully account for the observed patterns of morphological divergence. Both sets of *F*ST scores were uncorrelated with size or any *P*ST of shape (Mantel tests; $p > 0.05$ in all cases).

Interspecific Morphological Disparity

Drosophila koepferae and *D. buzzatii* were well separated and clearly discriminated in genital shape morphospace (Fig. 6). PC1 and PC2 jointly explained 70.2% of total shape variation (56.7 and 13.5% respectively). PC1 accounted for variation in thickness of the aedeagus and overall shape (e.g., with or without tip projection), thus summarizing the main interspecific differences. PC2 described variation in three main features of aedeagus shape, namely the orientation of the tip and development of its ventral projection, and the development of the ventral lobe of the aedeagus. Populations of *D. koepferae* were mainly differentiated in this latter shape dimension. VFE, the sole sympatric population of the species in our assay, was the most dissimilar population of *D. koepferae*. VFE

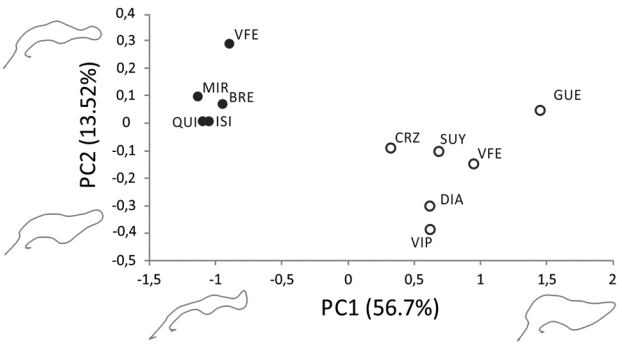

Fig. 6 Morphospace for natural populations of both species (*D. koepferae*: filled circles; *D. buzzatii*: empty circles). The first two principal components accounting for shape variation and the percentage of variance explained for each one of them (between parentheses) are depicted: PC1 versus PC2. Outlines of aedeagi by each axis represent genital shape variation accounted by each principal component. *VFE* Valle Fértil, *BRE* Brealito, *MIR* Miranda, *ISI* San Isidro, *QUI* Quilmes, *CRZ* Cruz del Eje, *SUY* Suyuque, *DIA* Diamante, *GUE* Guemes, *VIP* Vipos

moved away from the mean morphology of *D. buzzatii* along the PC2 but not along the PC1 where the greatest amount of interspecific variation was accumulated (Fig. 6). Populations of *D. buzzatii* displayed greater dispersion than populations of *D. koepferae* in both axes (Fig. 6). The level of morphological disparity among populations displayed by *D. buzzatii* was significantly higher than *D. koepferae* (Disparity test: 1.59 vs. 0.60 respectively, $p < 0.001$).

Discussion

Since the speciation event that set their independent evolutionary trajectories, *D. koepferae* and *D. buzzatii* took diverging paths in many aspects. The recent biogeographic expansion of the latter contrasts with the historically confined range of the former and their different ecological capabilities of host exploitation underlying this phenomenon are also strikingly different (Soto et al. 2008b, 2012, 2014; De Panis et al. 2016). Previous studies on *D. koepferae* have reported significant differentiation and genetic structuring in COI sequence as well as in microsatellites of these same populations with no evidence of recent demographic changes (Lipko 2013).

In the present study we found that male genital evolution could also be included in the list of contrasting features between these sibling species. Furthermore, our results also illustrate a frequently assumed but rarely confirmed fact: the striking morphological differences in genitalia could be rapidly produced in diverging lineages. Male genitalia of *D. koepferae* presented interpopulational differences in size but not in shape. Genital size diverged among populations in a degree compatible with expectations of neutral evolution. On the other hand, the levels of divergence were compatible with stabilizing selection in all shape dimension with respect to FST_{MS}. In contrast, for FST_{COI}, stabilizing selection was only supported for the main shape dimension (i.e., PC1), whereas for the remaining dimensions we found support for patterns of neutral evolution or even diversifying selection.

These divergent patterns of intraspecific shape variation suggest a complex scenario of antagonistic selective forces acting simultaneously on *D. koepferae*'s genital shape. Although it is an atypical pattern this should not be discarded a priori (see Simmons et al. 2009; Rowe and Arnqvist 2012; Simmons 2013; Brennan and Prum 2015; Anderson and Langerhans 2015).

It is worth mentioning that COI is known for its low levels of intraspecific variation (e.g., it is typically used in DNA barcoding) compared to other molecular markers loci regarded as good proxies of neutral variation as, for example, microsatellites or single-nucleotide polymorphisms (SNPs) (Leinonen et al. 2013). Therefore, COI variation in the present sample could be low, even lower than neutral, and thereby the patterns obtained for PC2–PC5 could be spurious. Besides, variation in mitochondrial genes can be reduced by Wolbachia-induced selective sweeps or demographic events as expansions or bottlenecks. The results of neutrality tests and populational structural analyses performed by Lipko (2013) over the same COI data ruled out these possibilities.

Both comparisons of shape variation against FST_{COI} and FST_{MS} show that the main shape dimension (i.e., PC1) has been diverging under a stabilizing selection regime. Additionally, the disparity analysis pointed out that *D. koepferae* has been exploring genital the morphospace more restrictively than its sibling. All in all, it seems likely that (at least) the most variable features of *D. koepferae*'s genitalia have been under stabilizing selection.

This selective regime could be the result of different processes, such as those of "Lock-and-Key" or Pleiotropy hypothesis or even classic sexual selection. Our current results are not entirely conclusive regarding the actual acting process (or processes) driving the genital evolution of *D. koepferae*. Nevertheless, there are some clues suggesting that the processes involved in the "Lock-and-Key" hypothesis could be underlying the observed pattern. For instance, previous comparative studies have shown that *D. buzzatii* presents host related phenotypic plasticity in genital morphology whereas *D. koepferae* possess a highly canalized genital development unaffected by the rearing medium (Soto et al. 2007). In contrast to the Pleotropy hypothesis, lack of phenotypic plasticity as the by-product of stabilizing selection acting to prevent heterospecific matings constitutes one of the predictions of "Lock-and-Key" (Arnqvist 1997; Kamimura 2012; Masly 2012). However, Soto et al. (2007) also found that *D. koepferae* shows high levels of genetic variation linked to the morphology of its genitalia, which could constitute evidence against the "Lock-and-Key" hypothesis. Further assays testing specific aspects of the competing hypothesis need to be conducted to identify the process (or processes) responsible for the observed pattern.

Morphological disparity among populations was two and a half times larger in *D. buzzatii* than in *D. koepferae*. This differential occupation of the genital morphospace was achieved after their divergence from a common ancestor, implying different rates of morphological evolution. These findings support previous results suggesting directional selective processes driving *D. buzzatii*'s genital evolution (Soto et al. 2013), which could then have achieved greater disparity than its putative selectively restrained sibling. Rapid divergence prompted by different scenarios of sexual selection is particularly well supported by comparative studies demonstrating that interspecific diversity of male genitalia is significantly higher in polyandrous species (where opportunity for postcopulatory sexual selection can be strong) than in monandrous species (Arnqvist 1998). *D. buzzatii* and *D. koepferae* greatly differed in their intraspecific levels of promiscuity and sperm competition, with postmating sexual selection stronger in *D. buzzatii* than in its sibling (Hurtado et al. 2013).

Noteworthy, the results of the morphological disparity analysis are congruent with patterns of genitalic morphological differentiation among species of the *D. buzzatii* cluster. The species of this group have a very similar aedeagi, the only exception being *D. buzzatii* which shows a

markedly different aspect both in shape and size (Fig. 1b). In this regard, Soto et al. (2008) showed that, while genitalia of the *D. serido sibling* set (all species except *D. buzzatii*) would have been diverging mainly under a drift-gene flow balance, *D. buzzatii*'s genitalia might have diverged from these species under directional selection.

Although patterns of intraspecific divergence differed between *D. koepferae* and *D. buzzatii*, the present data does not allow us to establish whether the acting process differ between species. For example, the evolutionary processes may be the same among populations and species but these processes may target different structures or operate in different ways (e.g., directional versus stabilizing selection) and thereby their outcomes in both species could differ. However, it is worth mentioning that previous studies suggested the existence of different scenarios of genital evolution and probably the evolution of different developmental networks in these sibling species (Carreira et al. 2006; Fanara et al. 2006; Soto et al. 2007). Thus, it seems safe to think that the different patterns observed here for *D. buzzatii* and *D. koepferae* may be a reflection of these underlying differences previously reported.

Our results show that an important fraction of *D. koepferae*'*s* male genital morphology has evolved under stabilizing selection, in contrast to its sibling *D. buzzatii* which has been diverging under directional selection, drift or by combination of both (Soto et al. 2013). According to our observations, regardless of the processes involved, *D. koepferae* and *D. buzzatii* have followed different evolutionary pathways since they split into different species. Although nowadays is widely accepted that genitalia can in principle evolve under different tempos and modes of selection in closely related species and even within the same species (Simmons 2013), few works have found unequivocal empirical evidence (e.g., Cordero and Eberhart 2005; Simmons et al. 2009; Rowe and Arnqvist 2012; House et al. 2013). In that sense, the present study constitutes an important contribution to the understanding of genital diversification in animals with internal fertilization.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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