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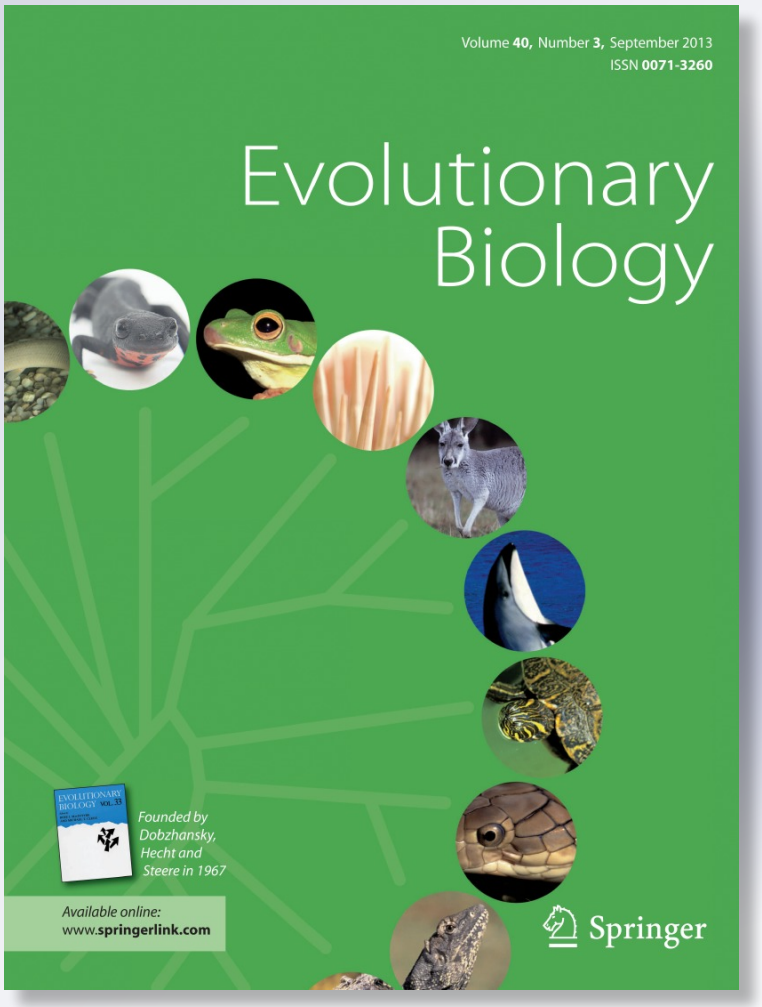
Evolutionary Biology
Evolutionary Biology

ISSN 0071-3260
Volume 40
Number 3

Evol Biol (2013) 40:395-407
DOI 10.1007/s11692-013-9223-x



Springer

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RESEARCH ARTICLE

Rapid Divergent Evolution of Male Genitalia Among Populations of *Drosophila buzzatii*

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Received: 14 November 2012 / Accepted: 1 February 2013 / Published online: 10 February 2013

Abstract Increasing evidence from multiple animal systems suggests that genital evolution and diversification are driven by rapid and strong evolutionary forces. Particularly, the morphology of male genital structures is considered to be among the fastest evolving traits in animal groups with internal fertilization. In this study, we investigated patterns of male genital variation within and between natural populations of the cactophilic fly *Drosophila buzzatii* in its original geographic distribution range in the Neotropics. We detected significant morphological differences among populations and distinguished five differentiated groups. Moreover, among population differentiation in genital morphology was associated with the degree of geographic isolation among populations and clearly contrasted with the general homogeneity detected for the putatively neutral mitochondrial gene *COI*. Integrating our present data with previous molecular population genetic surveys, our results suggest that male genital morphology has rapidly diverged after the recent demographic expansion that *D. buzzatii* has undergone in the arid zones of South America. Because the "lock and key" hypothesis failed to explain the present pattern, we explored alternative explanations for the observed pattern of genital diversification including drift-facilitated sexual selection.

Keywords Aedeagus · Chromosomal inversion · PST · Morphological evolution · Drift · *COI*

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Introduction

The degree of morphological differentiation among populations results from the interplay between selection, genetic drift, mutation and gene flow (Endler 1977). These processes affect the fate of genetic variation in the loci involved in the expression of morphological traits. In different populations, the environment may impose singular selective regimes on extant genetic variation promoting divergence in ecologically relevant (adaptive) traits among allopatric populations. Furthermore, even in the absence of adaptive differentiation, divergence among populations may merely reflect a balance between random genetic drift and gene flow. Gene flow tends to homogenize gene pools neutralizing or retarding adaptive and/or random differentiation (Slatkin 1987) whereas genetic drift, mutation and some types of natural selection may lead to genetic differentiation under limited gene flow (Hedrick 2005).

In the last few years there has been renewed interest in the interplay between natural selection and gene flow in studies of morphological variation (Crespi 2000; Schluter

2000; Hendry et al. 2002; Lenormand 2002; Saint-Laurent et al. 2003). Surveys of quantitative trait variation in natural populations revealed an inverse relationship between gene flow and the degree of among population differentiation for several adaptive morphological and behavioral traits in taxa as distinct as fishes (Hendry et al. 2002), amphibians (Storfer et al. 1999), birds (Smith et al. 1997), reptiles (King and Lawson 1995), insects (Sandoval 1994; Ross and Keller 1995) and arachnids (Riechert et al. 2001).

Reduced gene flow usually reflects population isolation or limited dispersal; however, it might also be indicative of reproductive barriers among groups independently of the presence of geographic barriers. Indeed, cause and consequence might invert if character divergence results in the formation of a reproductive barrier limiting gene flow among demes (i.e. ecological speciation; Schluter 1998; Lu and Bernatchez 1999; Coyne and Orr 2004).

The morphology of male genital structures is often cited as being among the fastest evolving traits in animal groups with internal fertilization (Kopp and True 2002; Hosken and Stockley 2004). Rapid morphological change might occur during speciation (McPeek et al. 2008). Rapid interspecific divergence and substantial amounts of intrapopulation genetic variation in genital morphology have been observed in several animal groups (Arnqvist 1997; Soto et al. 2007 and references therein) and selection (either natural selection or sexual selection) is often identified as the primary force. However, the underlying mechanisms and causal processes involved in genital evolution have not been fully established (McPeek et al. 2008).

The analysis of general patterns of intraspecific genitalic variation may be a useful tool for the critical assessment of alternative hypothesis aimed to explain the rapid evolution of male genital morphology (Arnqvist 1997; Soto et al. 2007). Discarding neutral evolution of genital structures, divergence in this kind of trait may be the consequence of genetic correlations with non genital traits (the hypothesis of pleiotropy) and/or sexual selection if conspecific populations enter independent coevolutionary processes underlying alternative inter and/or intra sexual conflicts (Arnqvist 1997). The "lock and key" hypothesis (Dufour 1844) states that genitalia evolve under selection for mechanical reproductive isolation and avoidance of hybridization. The two main predictions of this theory are a canalized development of male genitalia (i.e. a weak condition dependent expression of genitalic traits) and low levels of phenotypic and genotypic variation since genital traits are expected to be under strong stabilizing selection (Pomiankowski and Möller 1995; Arnqvist 1997). Although the "lock and key" hypothesis has often been discarded, recent studies renewed the interest on it (reviewed in Masly 2012). In fact, Wojcieszek and Simmons (2012) claim that the "lock and key" is the best explanation for the slow rate of genital divergence relative to expectations under neutrality in the millipede *Antichiropus variabilis*. Actually, this study is one of the few works approaching the evolution of male genital morphology from an intraspecific perspective, a not so frequently trodden path.

The aedeagus in *Drosophila* is a chitinous organ that consists of two dorsally fused hemipieces. The vast morphological diversity reported in several species groups of the genus makes the aedeagus a valuable diagnostic trait. This is especially true in groups in which members are difficult to distinguish using external morphology (Vilela 1983). Recent studies of male genital evolution in the *D. buzzatii* cluster (repleta group), an assemblage of seven closely related cactophilic species (Hasson et al. 2009), revealed that both genetic and ecological factors are determinants of intra- and interspecific variation. Actually, genital morphology exhibited plastic responses upon rearing on alternative host plants in *D. buzzatii*, *D. antonietae* and *D. gouveai* but not in *D. koepferae* (Soto et al. 2007, 2008). According to them, plastic responses along with allometric patterns of variation suggested incipient divergence in the reaction norms across these species. Moreover, the abundant phenotypic and genetic variation found in male genitalia in the *D. buzzatii* cluster argued against the "lock and key" hypothesis, whereas the condition dependence found (phenotypic plasticity in relation to cactus hosts) and the covariation with non-genitalic traits are in agreement with predictions of the pleiotropy hypothesis (Soto et al. 2007). Similar finding were independently reported for other species of *Drosophila* (Andrade et al. 2005, 2009). Briefly the pleiotropy hypothesis assumes that genital variation is largely neutral. Since genital and non-genital morphological traits are implicitly genetically correlated, changes of allele frequencies at loci pleiotropically affecting general morphology and genitalia may lead to rapid and arbitrary evolution of genital traits.

Drosophila buzzatii is, by far, the most studied species of the *D. buzzatii* cluster. In South America, it can be found from the arid lands of Northwestern Argentina and Southern Bolivia to the Atlantic coast of south and central Brazil (Hasson et al. 2009). Emergence records from naturally decaying cacti revealed that *D. buzzatii* breeds primarily on prickly pears (genus *Opuntia*) (Hasson et al. 1992) and due to the widespread use of these cacti as ornamental and semicultivated plants, *D. buzzatti* has reached a subcosmopolitan distribution (Hasson et al. 2009 and references therein). Relative to the other six species in the cluster, *D. buzzatii* has the most divergent genital morphology (Vilela 1983; Manfrin and Sene 2006; Soto et al. 2008), and is the only species that is not associated with columnar cacti as primary hosts (Hasson et al. 2009). Surveys of population structure have used inversion

polymorphism, allozymes and DNA sequence data (Fontdevila et al. 1982; Rodríguez et al. 2000; De Brito et al. 2002; Gómez and Hasson 2003; Piccinali et al. 2004, 2007); however, studies of intraspecific morphological diversity in natural populations are lacking.

In the present work, we examine within and among population variation in aedeagus morphology in natural populations of *D. buzzatii* throughout a wide area in South America. Our aim is to identify the evolutionary processes driving divergence in genital morphology among populations by conducting a within-species study. We determine whether divergence is compatible with expectations under random drift-gene flow balance or, alternatively with either natural or sexual selection. Furthermore, we evaluate alternative hypotheses that have been proposed to explain the evolution of genital morphology by contrasting predictions with observed patterns of genetic and phenotypic divergence.

Materials and Methods

Fly Collections and Morphological Quantification

Flies were collected with baited traps in 10 populations covering most of the species range in Argentina (Fig. 1), from 40 to 1,200 meters above sea level (Table 1). Collected males were preserved in 70 % ethanol. Aedeagi from males were dissected, mounted on microscope slides and flattened with cover slips using DPX (Sigma-Aldrich) as histological mountant. In order to prevent biases and to homogenize mounting errors among groups (e.g. possible slightly different angles of flattening) specimens were processed in random order and by the same person (IMS). Slides were photographed at 400× magnification with a digital camera mounted on a microscope. Aedeagus morphology was captured using elliptic Fourier descriptors (EFD) to quantify the outlines of the organ (for a detailed description see Soto et al. 2007). This methodology allows the description of the organ based on a set of coefficients corresponding to a polynomial function (a sum of ellipses) (Kuhl and Giardina 1982). Four elliptic Fourier coefficients for each harmonic (each basic sine or cosine function) were normalized for size, rotation and starting point of trace, so that three degrees of freedom disappeared in the normalization. Thus, the shape of the aedeagus was approximated using 117 coefficients of normalized EFDs, producing representations of the organ based on the shape of the outlines. Subsequently, a Principal Component Analysis (PCA) was performed using the variance–covariance matrix of the estimated coefficients in order to reduce dimensionality (Rohlf and Archie 1984) using only the principal components (PCs) that accounted for a proportion of morphological variation larger than 1/(Number of analyzed components). The excluded PCs are less likely to be biologically meaningful and might increase measurement error. The consequent PCs scores obtained for each male were considered as reordered morphological variables that allow the assessment of morphological variation (Iwata and Ukai 2002). For the morphological quantification, only the aedeagus was considered excluding the apodeme and the gonopods (Fig. 2). We measured the area of each outline and the square root of the area was used as a proxy of genital size.

The EFD analysis was performed using SHAPE v1.2 package (Iwata and Ukai 2002).

Differences among populations in genital size were evaluated by means of an ANOVA using the square root of the aedeagus area (size as in Garnier et al. 2005) as the dependent variable. Shape variation was analyzed by means of a MANOVA with significant PC scores as dependent variables. We also performed a MANCOVA using size as a covariate to analyze size-independent shape variation, since size differences may be due to uncontrolled variation in rearing conditions, and because genital shape may vary allometrically with size (Soto et al. 2007).

We also explored size variation along geographic gradients by means of regression analyses of population means of genital size on altitude and latitude. Regressions and analyses of variance were performed using Statistica (Stasoft Inc. 2001).

Additionally, we performed, a hierarchical clustering method (UPGMA) using the MDGC method (Method Dirienzo Guzman Casanoves) proposed by Valdano and Di Rienzo (2007) to determine overall genital differentiation among populations and to estimate the effective number of morphologically distinct groups. Briefly, this method is a hybrid technique that joins a hierarchical clustering method based on Mahalanobis distances with the principle of hypothesis testing for multivariate cases. This method is based on inferential statistics and is successful in determining the number of groups in a hierarchical cluster analysis. MDGC is recommended to resolve the number of clusters in these cases because, unlike other algorithms, it takes into account the fact that each treatment (population) is represented by a set of replicated observations (the specimens) (Valdano and Di Rienzo 2007). The MDGC test was run using Infostat software (Di Rienzo et al. 2009).

Interpopulational Genetic Differentiation

We estimated the degree of genetic differentiation among populations using two types of genetic markers: the second chromosome inversion polymorphism (for details see Soto et al. 2010) and sequence variation in the mitochondrial *cytochrome oxidase* subunit I (*COI*) gene.

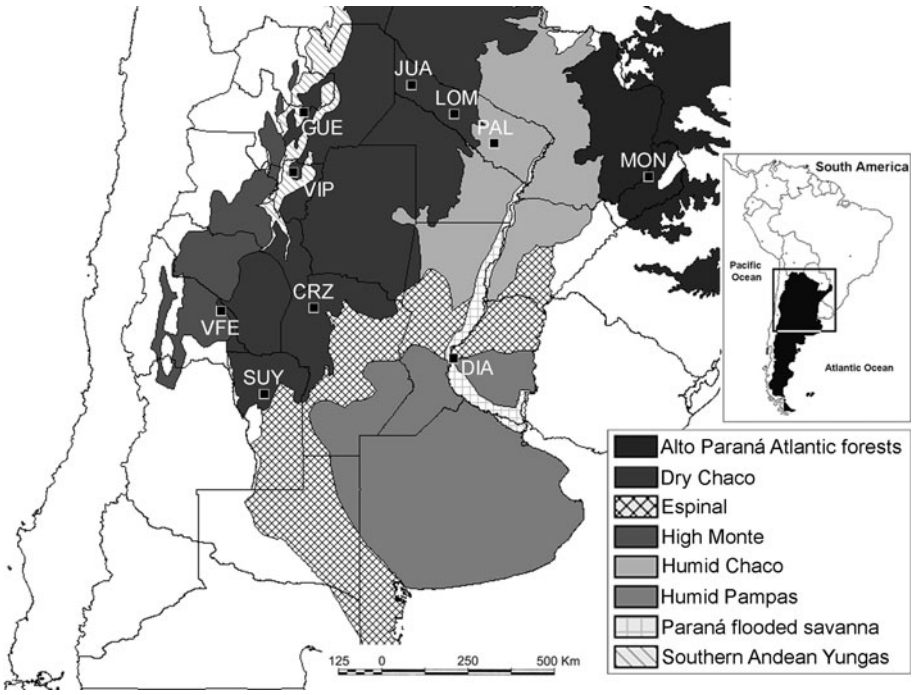

Fig. 1 Map showing the locations of the natural populations of *D. buzzatii* sampled and the phylogeographic regions according with Cabrera (1976). *Dotted line* marks the southernmost limit of the species distribution. Modified from Soto (2012)

Table 1 List of sampled localities along with geographical coordinates, altitude (meters above sea level), available cactus host (C: columnar, O: *Opuntia*) and the present of the sibling species: *D. koepferae*

Population	Acronym	Lat. (S)	Long. (W)	Altitude (m)	Cactus host	*D. koepferae* presence	N
Ingeniero Juárez	JUA	23°49′24″	61°52′09″	177	CO	n	39
Gral. Guemes	GUE	24°40′02″	65°03′53″	769	CO	n	7
Las Lomitas	LOM	24°42′07″	60°34′55″	133	CO	n	35
Palo Santo	PAL	25°33′37″	59°22′02″	92	CO	n	35
Vipos	VIP	26°29′13″	65°22′20″	1,000	CO	y	35
Montecarlo	MON	26°34′52″	54°43′38″	207	O	n	11
Cruz del Eje	CRZ	30°31′13″	64°48′23″	390	CO	n	18
Valle Fértil	VFE	30°38′47″	67°34′05″	1,189	CO	y	16
Diamante	DIA	32°02′12″	60°35′11″	40	O	n	11
Suyuque	SUY	33°07′29″	66°16′50″	1,008	CO	y	11

N number of males captured and analyzed

Second chromosome inversions affect several fitness related traits such as body size, developmental time, viability and longevity (Hasson et al. 1991, 1992; Fernández Iriarte and Hasson 2000). Second chromosome inversion frequencies in the populations studied in this paper were described elsewhere (Hasson et al. 1995; Soto et al. 2010). Among population differentiation for the inversion polymorphism was estimated using Nei's genetic distance (Nei 1972).

The matrilineal inheritance of the mtDNA and the high mutation rate make *COI* a useful tool to investigate population genetic structure. To this end, we sequenced a fragment of *COI* in 40 individuals derived from eight of the populations also characterized for genital morphology. Although flies from the localities of Suyuque and Palo Santo could not be included in the *COI* survey, all groups detected as phenotypically distinctive clusters by means of morphological analysis were represented in the survey. DNA was extracted from ethanol-preserved flies using QIAGEN DNA extraction Kit. Primers used for PCR and sequencing were the same as in De Brito et al. (2002). Purified PCR products using AccuPrep Purification Kit (Bioneer) were directly sequenced (Macrogen). Sequences were checked using Chromas lite v 2.0 freeware program (http://www.techelysium.com.au/chromas_lite.html) and aligned using MEGA v.5 (Tamura et al. 2011).

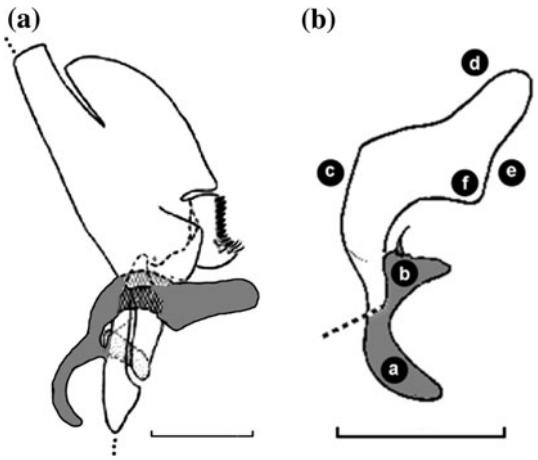

Fig. 2 **a** Schematic representation of the male genitalia of *Drosophila buzzatii* in left lateral view with a half emerged aedeagus (*shaded grey*). Modified from Vilela and Brito da Cunha (2006). **b** Schematic detail of aedeagus. The *shaded area* represents the internal portion of the organ that was excluded from 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). *Scale bar* 0.1 mm

We estimated among population differentiation using pairwise F_{ST} values. Analyses were conducted using the Maximum Composite Likelihood method in MEGA v.5. All codon positions were included in the analysis. Positions containing gaps and missing data were eliminated from the dataset using the complete deletion option. There were a total of 520 nucleotide positions in the final dataset.

Patterns of Genital and mtDNA Divergence

We investigated the processes driving genital evolution by comparing genetic (F_{ST}) and phenotypic (P_{ST}) differentiation across populations (Brommer 2011; Wojcieszek and Simmons 2012). Thus, for the group of eight populations for which we calculated F_{ST} we also estimated morphological divergence by computing P_{ST} values between pairs of populations. P_{ST} is analogous to Q_{ST} and quantifies the proportion of among-population genetic variance in quantitative traits (Spitze 1993). The use of P_{ST} instead of Q_{ST} has a disadvantage since the non-additive genetic variance, variance due to environmental factors and genotype by environment interactions, may give an inaccurate picture of additive genetic variance (Pujol et al. 2008). However, the estimation of Q_{ST} is not possible in most cases as it requires the rearing of all individuals included in the study in common-garden assays.

The null hypothesis of neutral morphological evolution (i.e. mutation/drift equilibrium) may be rejected either when P_{ST} is significantly greater or smaller than F_{ST}. The first scenario, $P_{ST} > F_{ST}$, may be the outcome of rapid morphological divergence among populations, and may reflect the hallmark of a directional process like sexual selection and/or sexual conflict (House and Simmons 2003; Hosken and Stockley 2004 but see McPeek et al. 2008). The alternative scenario, morphological divergence significantly lower than neutral divergence ($P_{ST} < F_{ST}$) may be interpreted as evidence of stabilizing selection governing genital evolution within populations.

We calculated P_{ST} using the formula:

$$P_{ST} = \sigma_B^2/(\sigma_B^2 + 2\sigma_W^2) \quad (1)$$

where σ_B^2 and σ_W^2 are the among and within population variance, respectively (Raeymaekers et al. 2007). As the complete original formula is (see Brommer 2011 for further discussion):

$$P_{ST} = (c/h^2)\sigma_B^2/[(c/h^2)\sigma_B^2 + 2\sigma_W^2] \quad (2)$$

by using Eq. 1, we are making the conservative assumption that the proportion of total variance that is assumed to be due to additive genetic effects across populations (c scalar) is equal to the heritability of the trait (h^2). Pairwise P_{ST} values were calculated for aedeagus size, and the first two principal components (PCs) of aedeagus shape (which describe more than 50 % of total phenotypic variation in genital shape). Variance components for P_{ST} estimation were obtained using one-way ANOVAs for each trait as the dependent variable and pairwise population combinations as the independent variable, using Statistica (Stasoft Inc. 2001).

We also tested for correlations between pairwise P_{ST} and pairwise F_{ST} values via Mantel tests (999 permutations), using the program Mantel v 1.19 (Cavalcanti, 2008). The same program was used for correlation analyses between matrices of phenetic (Squared Mahalanobis distances) and genetic (based on Nei's genetic distance in second chromosome inversion frequencies or F_{ST} for *COI* sequence variation) differentiation among populations and linear geographic distances among sites of collection.

To determine whether F_{ST} was significantly different from P_{ST} we calculated bootstrapped means with 95 % confidence limits for pairwise F_{ST} values and pairwise P_{ST} values (999 replications) for each trait, using the freely available software PopTools version 3.0.6 (Hood 2008).

In those cases in which P_{ST} differed significantly from F_{ST}, and before reaching any conclusion, the results were critically examined as these data may (probably) violate the assumption that the proportion of total variance due to additive genetic effects across populations (c scalar) equals the heritability of the trait (h^2). Since P_{ST} values were significantly larger than Fst we explored the robustness by comparing the statistical significance of the difference between P_{ST} and the neutral expectation in the range in which $c < h^2$ (see Brommer 2011 for a discussion). Thus,

using Eq. 2 we calculated the lower critical c/h^2 ratio where P_{ST} becomes equal to the upper confidence limit calculated for 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 the inference of selection.

Results

A total of 218 males were analyzed comprising ten natural populations of *D. buzzatii* (Table 1). Figure 2 shows a sketch of the aedeagus of *Drosophila buzzatii* in lateral view and the portion included in the morphometric study. The PCA of the variance–covariance matrix of the estimated EFD coefficients produced 11 significant components that jointly accounted for 91.3 % of the original shape variation. These components are those that explained a proportion of original variance greater than 1 divided by the total number of PCs. The first two components jointly accounted for 51.2 % of total shape variation. Figure 3 shows the distribution of the populations studied in the shape space delimited by the first two PCs. Two clusters of populations can be distinguished along the PC1 axis. The first, with negative scores, includes northeastern and central eastern populations (LOM, JUA, PAL, MON and DIA), while the second, with positive PC1 scores, comprises northwestern and central western localities (VIP, GUE, VFE, SUY and CRZ). Variation in shape generally involved changes in the dorsal margin ('*c*' in Fig. 2) of the organ (increasing values of PC1 and PC2 were associated with a more curved dorsal margin), and the relative thickness of the aedeagus (decreasing values of PC2 and increasing values of PC1 were related with thicker organs).

The ANOVA and the MANOVA revealed significant differences among populations in mean genital size (Table 2a) and shape (Table 2b), respectively. Shape differences among populations remained significant after removing size variation (the allometric component of shape variation) as confirmed by the results of the MANCOVA (Table 2c). Regression analyses of genital size on altitude ($p = 0.72$; $r = -0.05$) and latitude ($p = 0.59$; $r = -0.04$) were not significant.

Table 3 shows several pairwise distances (geographic, phenetic and genetic) calculated for the populations. The phenogram constructed using Mahalanobis distances among populations showed a clustering pattern (Fig. 4) coincident with a broad geographic structuration. The MDGC test found six significant groups ($\alpha = 0.05$): four populations (MON, GUE, CRZ and DIA) remained as distinctive morphological groups, while the remaining populations formed two different clusters, one comprising western populations (VIP, SUY and VFE) and the other encompassing northeastern localities (LOM, JUA and PAL; Fig. 4). These localities formed a more inclusive cluster with Diamante (DIA), which was differentiated from the other clusters. The populations showing the most divergent male genital shapes were Güemes (GUE) and Montecarlo (MON).

According to the results of the Mantel Test, only the phenetic and geographic distance matrices (Table 3) were significantly correlated (Approximate Mantel t test = 3.293, $r = 0.52$; $p = 0.005$; Table 5), providing evidence for a standard isolation-by-distance pattern of male genital shape.

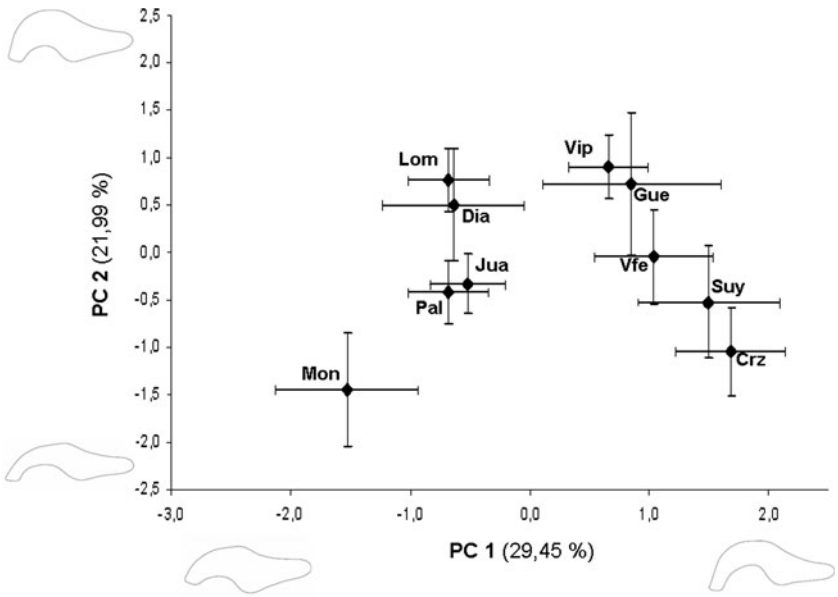

Fig. 3 Plot of mean shape scores (and standard errors) for each population. The first two principal dimensions accounting for shape variation (PC1 and PC2) and the percentage of variance explained for each one of them (between *parentheses*) are depicted. Outlines of aedeagi by each axis represent genital shape variation accounted by each principal component

Table 2 Sources of variation in male genital morphology

Sources of variation				
Size	*df*	MS		F
(a)				
Population	9	2,260		3.39*
Error	208	666		
Shape	*df* Effect	*df* Error	Wilk's value	*F*
(b)				
Population	99	1,407.42	0.20	3.66*
(c)				
Size	11	197	0.64	9.99*
Population	99	1,400.69	0.21	3.54*

Results of the ANOVA testing for differences in size (a) among natural populations of *D. buzzatii* and multivariate analyses testing for total shape differences (MANOVA), (b) and the non allometric component of shape variation (MANCOVA, (c)

df degrees of freedom, *SS* sum of squares, *MS* mean squares. * $p < 0.001$

We estimated population genetic structure using second chromosome inversion frequencies and *COI* sequence variation. The raw data for the inversion polymorphism was reported elsewhere (Soto et al. 2010) and the matrix of Nei's genetic distance between pairs of populations is given in Table 3.

The analysis of *COI* sequence variation revealed a G + C content of 0.326 and 46 variable sites (10 of which were singletons) distributed in 12 haplotypes. Haplotype diversity (*Hd*) was 0.686 ± 0.078 and nucleotide diversity (π) 0.015. The among-population differentiation for *COI* was estimated using F_{ST}. The analysis of the *COI* dataset revealed a very weak population structure (similar results were obtained using a larger dataset including more sampling localities P. Lipko and E Hasson, unpublished results).

Matrices of genetic distance obtained for both the inversion polymorphism and *COI* were not correlated with each other or to the phenetic or geographic distances matrices ($p > 0.05$ in all cases, Table 5).

We explored if drift-gene flow balance could explain the observed pattern of morphological divergence among populations in male genitalia. Table 4 contains the F_{ST} and P_{ST} values for both genital size and shape. In Fig. 5 we present the plots of P_{ST} for genital size and the first two principal components (PC1 and PC2) describing shape variation on F_{ST}, which may be used as surrogate of expectations under neutrality. According to Mantel tests only the P_{ST} matrix constructed with the first shape variable (PC1; $r = 0.42$, $p < 0.05$) was significantly correlated with F_{ST} but not the matrices constructed with genital size

Table 3 Geographic (in kilometers; below diagonal), phenetic (Mahalanobis distances, above diagonal) and genetic (Nei's distance, between parentheses above diagonal) distances among localities sampled

	Cruz del Eje	Güemes	Suyuque	Valle Fértil	Vipos	Las Lomitas	Ing. Juarez	Palo Santo	Montecarlo	Diamante
Cruz del Eje	0	2.88 (0.00)	1.39 (0.01)	1.89 (0.02)	2.15 (0.02)	2.84 (0.71)	2.18 (0.71)	2.3 (0.71)	3.61 (0.06)	3.39 (0.02)
Güemes	654	0	2.38 (0.02)	2.12 (0.03)	2.01 (0.01)	2.33 (0.80)	2.43 (0.80)	2.27 (0.80)	3.56 (0.05)	2.6 (0.03)
Suyuque	319.3	944.06	0	1.46 (0.00)	1.62 (0.04)	2.38 (0.63)	2.16 (0.63)	2.23 (0.63)	3.29 0.10)	3.07 (0.02)
Valle Fértil	259.7	705.12	299.91	0	1.48 (0.06)	2.23 (0.50)	1.6 (0.50)	1.73 (0.50)	3.2 (0.13)	2.64 (0.01)
Vipos	450.3	199.47	743.51	503.31	0	1.54 (1.11)	1.77 (1.11)	1.66 (1.11)	3.43 (0.02)	2.32 (0.08)
Las Lomitas	768.51	456.67	1,080.94	942.62	516.55	0	1.5 (0.00)	1.45 (0.00)	2.73 (1.83)	2.16 (0.41)
Ing. Juarez	798.32	338.96	1,115.74	933.8	458.65	161.17	0	0.85 (0.00)	2.45 (1.83)	1.88 (0.41)
Palo Santo	764.6	580.89	1,068.87	970.21	608	156.1	323.33	0	2.55 (1.83)	2.21 (0.41)
Montecarlo	1,077.9	1,062.77	1,320.63	1,320.5	1,057.03	620.44	780.8	478.12	0	2.76 (0.17)
Diamante	425.05	929.95	545.28	672.29	769.76	810.13	922	725.18	832.69	0

Table 4 Genetic and phenotypic differentiation among populations estimated by means of F_{ST} values (below diagonal) and P_{ST} values (above diagonal) for genital size and the first two shape principal components (PC1 and PC2) respectively

	Juarez	Guemes	Lomitas	Montecarlo	Cruz del Eje	Ensenada	Valle Fértil	Vipos
Juarez		0.75/0.14/0.65	0.25/0.83/0.48	0.52/0.77/0.51	0.72/0.86/0.71	0.85/0.71/0.87	0.16/0.57/0.57	0.40/0.38/0.05
Guemes	0.00		0.70/0.77/0.76	0.04/0.82/0.15	0.75/0.61/0.73	0.02/0.70/0.03	0.49/0.13/0.09	0.71/0.37/0.56
Lomitas	0.00	0.00		0.64/0.17/0.73	0.80/0.95/0.46	0.83/0.05/0.92	0.00/0.89/0.75	0.60/0.55/0.21
Montecarlo	0.00	0.00	0.00		0.03/0.93/0.74	0.76/0.02/0.56	0.44/0.85/0.01	0.12/0.53/0.44
Cruz del Eje	0.74	0.72	0.74	0.71		0.85/0.91/0.89	0.64/0.47/0.76	0.36/0.89/0.60
Ensenada	0.50	0.30	0.50	0.19	0.73		0.66/0.80/0.36	0.80/0.43/0.79
Valle Fértil	0.49	0.47	0.49	0.46	0.00	0.49		0.41/0.71/0.52
Vipos	0.00	0.00	0.00	0.00	0.40	0.05	0.12	

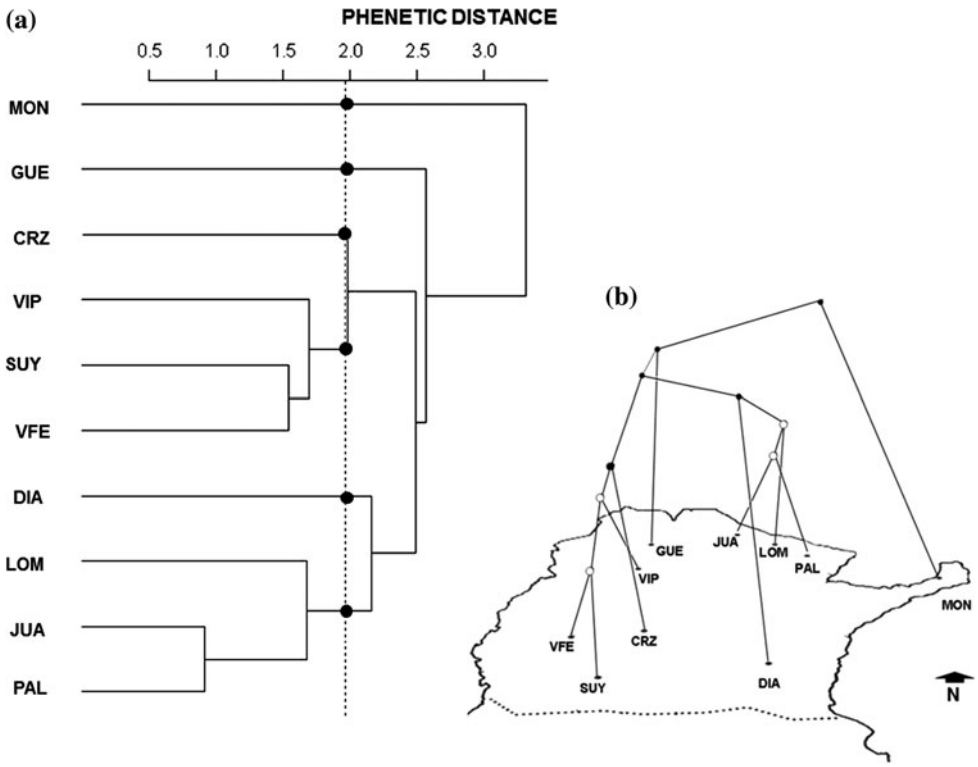

Fig. 4 **a** Phenogram of male genital shape constructed using Mahalanobis distances among populations. Black nodes group locations with significant differences in genital morphology according to the cut-off criterion of the MDGC test (*horizontal dotted line* in the phenogram, α value = 0.05, see text) and; **b** its projection plotted onto the distribution map (Branch lengths are not proportional). *Dotted line* marks the southernmost limit of *D. buzzatii* distribution

Table 5 Correlation coefficients obtained by Mantel tests of the Geographic (distances in km.), phenetic (Mahalanobis distances), inversion polymorphism (Nei distances) and *COI* divergence (F_{st} values) distances among populations

	Geographic	Phenetic	Inversion
Phenetic	r = 0.52*	_	
Inversion	r = −0.018	r = −0.135	_
COI	r = 0.287	r = 0.203	r = −0.175

Significant correlations (999 permutations, probability random Z < observed Z) are denoted by an asterisk if $p > 0.05$

nor the second shape variable (r values <0.28; both p values >0.05). However, most points fell below the line defined by the values expected under neutrality, i.e. equality of P_{ST} and F_{ST} for size and shape variables (Fig. 5), suggesting that mutation-drift balance is not a sufficient explanation for the patterns of morphological divergence observed.

Means and 95 % confidence limits of pairwise F_{ST} and P_{ST} values are presented in Table 6. All morphological variables presented P_{ST} means greater than mean F_{ST} and outside the 95 % confidence interval, suggesting that male genital morphological differentiation (both size and shape) exceeds what may be expected on the basis of genetic differentiation at neutral loci. These results suggest that morphological differentiation among populations has been the result of some type of directional selection. This was evident even for the P_{ST} matrix for the first shape variable that was the only one significantly correlated with the F_{ST} matrix.

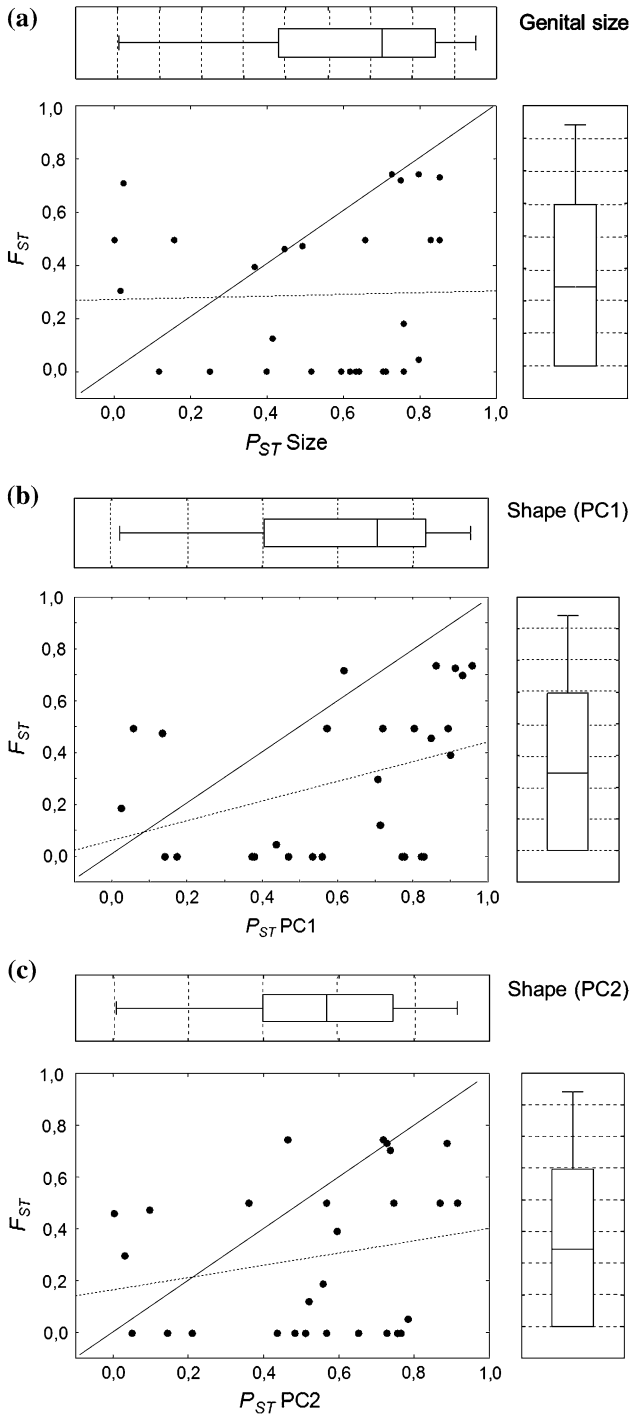

Fig. 5 Plots of phenotypic (P_{ST}) differentiation compared to putative neutral genetic differentiation (F_{ST}) among populations for: **a** male genital size; and the first two shape variables: **b** PC1 and **c** PC2. *Solid lines* represent $P_{ST} = F_{ST}$ as expected by neutral evolution. *Dotted lines* are the actual linear trend. Diagrams along the axis represent the maximum and minimum values (*spreads*), the 25–75 % percentiles (*boxes*) and the mean value (*line within boxes*)

However, before drawing any further conclusions, these results must be critically examined since these data may violate the assumption that the proportion of total variance due to additive genetic effects across populations (c scalar) equals the heritability of the trait (h^2). In fact, if P_{ST} exceeds neutral expectations, investigation of the robustness of conclusions regarding selection requires comparing the statistical significance of the difference between P_{ST} and the neutral expectation in the range in which $c < h^2$ (Brommer 2011). We calculated the lower critical c/h^2 ratio where P_{ST} becomes equal to the upper confidence limit of F_{ST} (0.291). The lower the critical c/h^2 ratio is for a statistically significant difference between P_{ST} and the neutral expectation, the more robust the inferences of selection (considering P_{ST} as a good proxy of Q_{ST}). The critical PST values were 0.36, 0.27 and 0.36 for size, PC1 and PC2, respectively (Table 6) indicating that the additive genetic component among populations had to be nearly 1/3 of the heritability within populations to accept drift and discard directional selection as the driving force of genital evolution.

Table 6 Means and upper and lower 95 % confidence limits for pairwise F_{ST} and P_{ST} estimates, following bootstrapping

	Mean	Lower 95 % Confidence limit	Upper 95 % Confidence limit	Critical c/h^2 Value
F_{ST} (*COI*)	0.288	0.285	0.291	
P_{ST} size	0.536	0.533	0.539	0.36
P_{ST} shape (PC1)	0.602	0.599	0.605	0.27
P_{ST} shape (PC2)	0.533	0.530	0.537	0.36

Calculation of critical c/h^2 ratio following Brommer (2011)

Discussion

Our study shows that natural populations of *D. buzzatii* are differentiated for both size and shape of male genitalia but not for putative neutral markers as the *COI* gene. Moreover, patterns of morphological and genetic differentiation among populations were uncorrelated and suggest that male genital morphology has diverged rapidly as a result of either natural or sexual selection.

In a theoretical classic prelude to allopatric speciation, variation fuels evolutionary change and differentiation among demes subjected to selective and/or stochastic forces. Thus, the study of intra- and interspecific variation are valuable approaches not only for the inference of the evolutionary history of species but also as identifiers of the actual evolutionary units in nature (Crandall et al. 2000).

Cactophilic *Drosophila* are characterized by the intimate association between flies and cactus hosts. Such close association to specific rearing substrates imposes a patchy distribution that may affect population connectedness (Manfrin and Sene 2006). Although this effect may vary

across species (Markow and O'Grady 2006), in most cases, environmental factors and geography set the scenario for morphological evolution (Polihronakis Richmond et al. 2012 and references within).

In the present work we found that natural populations of this species are phenotypically differentiated in both genital size and shape (the latter being explained by an allometric as well as non-allometric component). Moreover, we detected an association between genital differentiation and geographic distance among populations implying that populations located in closer proximity tended to show more similar morphology than more distant populations. Our analysis defined six morphological clusters (Fig. 4). However, our results revealed some inconsistencies to the overall correlation between phenetic similarity and geographic distance. The first is the observation that Güemes and Vipos (GUE and VIP) populations are morphologically more differentiated than would be expected on the basis of their geographic proximity and genetic similarity (measured in terms of differentiation in second chromosome inversion frequencies or F_{ST} values). Thus, other factors such as the specific host cactus in which flies develop are known to affect male genital morphology in *D. buzzatii* (Soto et al. 2007) and also the presence of closely related (competing) species may promote further evolution in genital morphology (Soto 2012) contributing to the overall pattern of morphological divergence. Interestingly, *D. buzzatii* is sympatric with its sibling *D. koepferae* in Vipos, Suyuque and Valle Fértil. Actually, the genitalia of *D. buzzatii* males is smaller and more differentiated in shape as compared to males derived from allopatric populations, suggesting some kind of character displacement (Soto 2012). It is also worth noting that in its global distribution, *D. buzzatii* could potentially be in sympatry with almost all other species of the cluster (Hasson et al. 2009).

The second inconsistency is illustrated by the extreme morphological differentiation of Montecarlo (MON) with respect to neighbouring localities in Northeastern Argentina sampled in the present study. This particular case could be explained in terms of historical and adaptive arguments. Montecarlo is the only population close to the Paranaense rain forest and, thus, it may be subject to different selective pressures relative to flies inhabiting more xeric environments. In addition, flies living in this area breed and feed on the rotting cladodes of the introduced and cultivated *Opuntia ficus indica* which has been introduced in historical recent times, suggesting a recent colonization event. Adaptation to this exotic host plant may explain the extreme morphological and chromosomal differentiation (Soto et al. 2010) relative to neighbouring populations. These observations raise interesting questions, since geographic distance does not account for the entire pattern of morphological differentiation.

In *D. buzzatii*, geographic differentiation has also been reported for the inversion polymorphism (Hasson et al. 1995; Rodríguez et al. 2000), and the available evidence indicates that performance of the carriers of different inversion karyotypes is dependent on the cactus host where the flies developed (Fernandez Iriarte and Hasson 2000; Fernandez Iriarte et al. 2002). However, patterns of among population differentiation in inversion frequencies, life history traits and genital morphology (Hasson et al. 1995; Fanara et al. 2006) are in sharp contrast with the lack of genetic structure for mtDNA sequence variation (Rossi et al. 1996) and synonymous variation at three nuclear genes linked to the polymorphic second chromosome (Gómez and Hasson 2003; Piccinali et al. 2004, 2007). If we accept the well founded proposal that *D. buzzatii* has undergone a recent population expansion based on population genetic surveys using nuclear and mitochondrial markers (Rodríguez et al. 2000; Gómez and Hasson 2003; Laayouni et al. 2003; Piccinali et al. 2004), our present data are consistent with the hypothesis of selective forces shaping variation in male genitalia, life history traits and inversions.

We also compared the pattern of morphological differentiation among populations with the pattern inferred from the survey of nucleotide variation in the mitochondrial *COI* gene. Most pairwise F_{ST}s were lower than 0.30, suggesting low levels of neutral genetic divergence that might be interpreted as the result of recent high levels of gene flow and/or a recent expansion of the species range (Gómez and Hasson 2003; Laayouni et al. 2003; Piccinali et al. 2004).

Thus, random differentiation at presumptively neutral traits had not enough time to occur since the demographic expansion in this widespread species, in contrast to our present observations for genital morphology. Both size and shape of the aedeagus showed a pattern consistent with rapid divergence among populations as if sexual selection and/or other sexually related selective processes were operating. All P_{ST} estimates were consistently higher than measures of genetic differentiation for the putatively neutral marker *COI*, failing to comply with the expectation of proportionality with the F_{ST} matrix as expected under random drift-mutation balance. This pattern of morphological differentiation in male genital morphology is in sharp contrast with a recent report in the millipede *A. variabilis* in which genital divergence among populations seems to be under strong stabilizing selection (Wojcieszek and Simmons 2012).

We explored the robustness of our inference of selective differentiation by examining alternative scenarios other than the null assumption of additive genetic effects across populations equal to the heritability of the traits ($c/h^2 = 1$). Brommer (2011) considered that a $P_{ST} > F_{ST}$ provides a robust inference of selective differentiation if the critical

value of c/h^2 not lower than 0.1. We calculated the critical values of the 95 % confidence interval of c/h^2, and the values obtained (0.27–0.36) suggest that additive genetic effects across populations should be lower than 30 % of the additive effects within populations to accept the null hypothesis of random morphological differentiation. Whitlock (2008) considered that given the usual values of heritability (often less than 0.5), the P_{ST} is downwardly biased as a Q_{ST} proxy. Thus, our P_{ST} estimations may be considered as conservative approximations Whitlock (2008) also noted that in studies including 10 or more populations (as in our case) the critical value to consider that the estimated Q_{ST} is significantly different from neutral divergence should be twice the mean F_{ST}, as we observed for some important aspects of genital shape (PC 1).

We are aware that, when quantitative variation is measured using wild phenotypes, environmental effects may obscure the actual levels of quantitative divergence. If phenotypic divergence reflects mainly plastic responses to different environments population divergence can be overestimated or underestimated in cases in which environmental effects reduce phenotypic variation despite high levels of genetic divergence (Leinonen et al. 2008). These authors performed a meta-analysis of quantitative variation uncovering a wide variety of traits (morphological, life history and behavioral) in groups as diverse as plants, invertebrates, vertebrates and fungi and demonstrated that studies based on information from wild phenotypes do not tend to yield higher estimates of quantitative divergence than studies based on common garden experiments.

Concerning the mechanisms involved in the divergence of genital morphology, our findings are congruent with the sexual selection model for genital evolution that predicts genital structures evolving under continuous directional selection, and thus experiencing continuous change over time (Arnqvist 1997; Hosken and Stockley 2004).

Moreover, our results are compatible with the recently proposed evolutionary process "coupled drift" (Tazzyman and Iwasa 2010). In this scenario, females may discriminate potential mates during copulation by their genital morphology (Jagadeeshan and Singh 2006). With female preference as the leading trait evolving by drift, the evolution of the follower trait (male genital morphology, forced by selection to match the mean female preference) would also be dictated by drift on the leader trait. Thus, coupled evolution would be characterized by a pattern resembling random drift at the among population level, but in a shorter evolutionary timespan due to the acceleration imposed by sexual selection. Uyeda et al. (2009) simulated different conditions of evolution of sexual isolation and showed that drift could promote rapid speciation by sexual selection. Actually, the role of drift as a "process amplifier" may be applicable to a broad range of assumptions, in some cases, working along selective processes on female preferences.

Although further studies investigating female mating behaviour and preference are necessary to confirm our interpretation, our present study along with previous surveys of population genetic structure unveiled a suggestive pattern. Patterns of population structure for the chromosomal polymorphisms and quantitative traits (life-history and morphological traits) are concordant with the operation of strong selective forces as suggested by the association with ecological and relevant environmental variables. The present study shows that male genitalia should be included in the list of divergent characters which are in frank opposition to the weak neutral genetic divergence uncovered by molecular markers, suggesting that divergence in male genital morphology was either rapid and/or operated despite the homogenizing action of gene flow.

Acknowledgments The authors wish to thank F. F. Franco and M. Polihronakis Richmond for helpful discussions in early stages of this investigation and in the drafting of the manuscript respectively. We are also grateful to two anonymous reviewers whose insightful advice and corrections helped to greatly improve the original manuscript. This work was supported by grants of Universidad de Buenos Aires, ANPCyT and CONICET. P. L., F. M. and E. M. S. are recipients of postgraduate and postdoctoral scholarships of CONICET respectively. I. M. S., V. P. C. and E. H. are members of Carrera del Investigador Científico (CONICET).

Conflict of interest The authors also have no conflict of interest to declare.

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