Rapid divergence of courtship song in the face of neutral genetic homogeneity in the cactophilic fly *Drosophila buzzatii*

PATRICIA P. IGLESIAS[1,2]*[†], IGNACIO M. SOTO[1,2], EDUARDO M. SOTO[1,2], LUCIANO CALDERÓN[3], JUAN HURTADO[1,2] and ESTEBAN HASSON[1,2]*

[1]*Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Ecología, Genética y Evolución, Buenos Aires, Argentina*
[2]*CONICET-Universidad de Buenos Aires, Instituto de Ecología, Genética y Evolución de Buenos Aires (IEGEBA), Buenos Aires, Argentina*
[3]*CONICET-Instituto de Biología Agrícola de Mendoza (IBAM), Mendoza, Argentina*

A wide spectrum of song complexity has been documented in a broad range of animal taxa. However, the underlying processes shaping acoustic differences are still poorly understood. Empirical and theoretical studies suggest that different song parameters may be subject to different tempos and modes of evolution, resulting in a complex combination of stabilizing and directional selection that change among populations and through time. Nonetheless, the contribution of genetic drift to acoustic divergence is largely unexplored. In this study, we used the Q_{ST}–F_{ST} approach to investigate the relative role of selective and neutral processes in the evolution of courtship song in the cactophilic fly *Drosophila buzzatii*. We quantified variation in courtship song among 12 populations using flies reared under common-garden conditions. We found among-population divergence in courtship song parameters, but genetic homogeneity and no population structure. Using both mitochondrial DNA and microsatellite markers, we found evidence consistent with a role for directional selection causing song divergence. We discuss the statistical difficulties of detecting stabilizing selection as opposed to directional selection in our data. The role of song parameters in mate recognition and mate choice, and the role of sexual selection in song elaboration are also discussed.

ADDITIONAL KEYWORDS: common-garden – divergent selection – microsatellites – mtDNA – Q_{ST}–F_{ST}

INTRODUCTION

Songs are a key component of many animal courtship displays, playing an important role in intersexual communication (Podos & Patek, 2015). Due to their diversity and high rate of evolution, courtship songs are a good model for evolutionary studies of sexual signals. A wide spectrum of song complexity has been documented within several groups, including frogs (Bevier *et al.*, 2008), birds (Catchpole & Slater, 2003), insects (Drosopoulos & Claridge, 2005), fishes (Amorim, 2006) and even mammals (Thinh *et al.*, 2011). However, the underlying processes shaping these acoustic differences are still poorly understood. Evidence suggests that evolution of sexual signals is highly complex in nature and is rarely explained by a single selective force (Bentsen *et al.*, 2006; Gerhardt & Brooks, 2009; Oh & Shaw, 2013). The expected trend is for characters subject to strong directional selection to exhibit rapid variation, whereas components under stabilizing selection to exhibit evolutionary stasis (Oh & Shaw, 2013). Nonetheless, the relative contribution of selection and drift to the acoustic divergence is largely unexplored (Wilkins, Seddon & Safran, 2013).

Empirical data suggest that different courtship song parameters may be subject to different tempos and modes of evolution, resulting in a complex combination of multivariate stabilizing and directional selection that changes among populations and through time (Catchpole & Slater, 2003; Bentsen *et al.*, 2006;

*Corresponding authors. E-mail: patricia.p.iglesias@gmail.com; ehasson@ege.fcen.uba.ar
†Present address: Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" – CONICET, Buenos Aires, Argentina.

Klappert *et al.*, 2007; Gerhardt & Brooks, 2009; Oh & Shaw, 2013; Tanner *et al.*, 2017). Sexual selection by female choice has often been invoked to explain the evolution of elaborated traits (Darwin, 1859, 1871; Andersson, 1994). It has become widely accepted that song elaboration in some birds and frogs evolved under the pressure of sexual selection (Collins *et al.*, 2009; Akre & Ryan, 2010; Okanoya, 2015). In addition, although selection for mate recognition is frequently associated with stabilizing selection (see Templeton, 1979; Gerhardt, 1991; Paterson & Shane, 1993; Arnqvist, 1997; Shaw & Herlihy, 2000; Ferreira & Ferguson, 2002; Hosken & Stockley, 2004; McPeek *et al.*, 2008, 2011; Arbuthnott *et al.*, 2010; Wojcieszek & Simmons, 2012), species interactions in sympatry can drive divergence in these parameters through reproductive interference (Dobzhansky, 1940; Blair, 1955; Howard, 1993). The distribution of signals in the male population reflects variation among females in their mean preference and in the rate at which the probability of response declines for signals that deviate from each female's mean preference (i.e. preference windows; Butlin, 1993). Both mean preference and preference window variation may have genetic and environmental components (Butlin, 1993). In this context, high levels of male signal variation within populations could be due to female mean preferences close to the mean male signal and wide preference windows, encompassing most of the distribution of signals in the male population (resulting in weak stabilizing selection; Butlin, 1993). Nonetheless, the maintenance of high levels of genetic variation for female mean preference within populations could also mimic a stabilizing selection scenario.

Geographical patterns of genetic variation can provide insight into the processes that drive song evolution. Comparing the level of genetic differentiation for quantitative traits (Q_{ST}) and neutral loci (F_{ST}) is a valuable approach to differentiate between genetic drift and selection as drivers of complex polygenic trait variation (Whitlock, 2008; Hasson *et al.*, 2013; Leinonen *et al.*, 2013). This technique allows us to assess whether the observed pattern of song variation is compatible with a scenario of: (1) only genetic drift ($Q_{ST} = F_{ST}$), (2) directional selection ($Q_{ST} > F_{ST}$), as is expected for parameters involved in sexual selection, or (3) stabilizing selection ($Q_{ST} < F_{ST}$), as is expected for parameters involved in mate recognition (Wojcieszek & Simmons, 2012; Leinonen *et al.*, 2013).

Fruit flies of the *Drosophila repleta* group exhibit great song diversity (Ewing & Miyan, 1986). During the mating ritual, male flies vibrate their wings producing a species-specific pattern of airborne vibrations called 'courtship song'. These songs consist of short waveforms (i.e. pulses) which are usually repeated to form trains. Pulse trains can be characterized by the carrier frequency of the pulses (CF) and the time interval between pulses (interpulse interval, hereafter IPI). Variation of the acoustic signal ranges from species producing only one type of song to species producing two types (i.e. varying in IPI and/or CF; Ewing & Miyan, 1986; Oliveira *et al.*, 2013). Independent evidence supports the idea that the performance of two different songs is the plesiomorphic condition (i.e. each song having a unique characteristic IPI value; Ewing & Miyan, 1986). The loss of one song type and/or the elaboration of the song pattern have been involved in the evolution of courtship song within this group (Ewing & Miyan, 1986). Thus, some songs have diverged from the plesiomorphic condition and have a bimodal distribution of IPIs, called doublet pulses (i.e. alternate pulses with long and short IPIs in the same song), and in some species there is a tendency for the secondary song to be run together with the primary song in a single burst, called composite burst (Ewing & Miyan, 1986; Oliveira *et al.*, 2013; Iglesias & Hasson, 2017).

Within the *D. repleta* group, the cactophilic fly *D. buzzatii* Patterson & Wheeler, 1942 is an excellent model species for addressing the relative importance of mate recognition, sexual selection and genetic drift in driving the evolution of complex songs. Courtship song in *D. buzzatii* consists of simple bursts of primary song with doublet pulses, and composite bursts (Iglesias & Hasson, 2017: fig. 6). Previous work has demonstrated that courtship song is part of the mate recognition system in this species (Iglesias & Hasson, 2017). The evolutionary dynamics expected for a trait that is part of the mate recognition system is to slow the rate of trait change after the time of speciation (McPeek *et al.*, 2008). However, doublet pulses and composite bursts are the most elaborated features; they might therefore be under sexual selection. In that case, we would expect song parameters related to these features to change continuously over time because of the directional selection pressures imposed by females (McPeek *et al.*, 2008). In addition, *D. buzzatii* is spread over a wide geographical range in South America in close association with the cactus hosts used as feeding and breeding sites (especially prickly pears of the genus *Opuntia*). Such close association to specific host plants imposes a patchy distribution that may affect population dynamics.

In this study, we examined courtship song variation of *D. buzzatii* throughout a wide area in Argentina and asked what processes best explain the observed pattern. First, we quantified variation in courtship song using flies reared under common-garden conditions to control for potential environmental effects. We also quantified the extent of neutral genetic differentiation among populations by means of both mitochondrial DNA (mtDNA) and microsatellite markers. Finally, we used the Q_{ST}–F_{ST} approach to investigate the relative

roles of selective and neutral processes in the evolution of courtship song.

MATERIAL AND METHODS

SAMPLE COLLECTION

Flies were collected with baited traps from 12 populations throughout a wide area in Argentina (Fig. 1; Table 1) and brought to the laboratory. Because species of the *D. buzzatii* cluster are cryptic, females were allowed to lay eggs and the male progeny were inspected to determine the species (Vilela, 1983). Eight to 15 isofemale lines were established per population and used in the experiments described below.

Flies were raised on standard *Drosophila* medium for four generations at 25 ± 1 °C under a 12-h light: 12-h dark cycle. As the expression of quantitative traits is generally plastic with respect to environmental effects (West-Eberhard, 2003), experimental flies were raised under common-garden and controlled-density conditions. Thus, batches of 40 first-instar larvae were seeded in vials containing 6 mL of 'semi-natural' medium prepared with fresh cladodes of the cactus *Opuntia ficus indica*. We used this cactus species because *D. buzzatii* uses prickly pears as primary hosts in nature and *O. ficus indica* is the most widespread

species in the distribution sampled. For preparation of 'semi-natural' medium, cactus tissues were mixed in a blender and 1% dehydrated commercial yeast (*Saccharomyces cerevisiae*) and 0.8% agar were added. Dehydrated commercial yeast was used as protein source and agar to control the consistency of media. Once prepared, vials were autoclaved.

The courtship song of one 5-day-old male per isofemale line was recorded and right wings were removed with a pair of microsurgical scissors from the base of the wing and measured (see below). In addition, one male per isofemale line was conserved in ethyl alcohol 96% to be used in genetic analysis.

WING SIZE QUANTIFICATION

Some song parameters have been found to covary with size in insects (e.g. carrier frequency; Cocroft & De Luca, 2006); thus, we tested for correlations between wing size (which is also used as a proxy for body size; Robertson, 1962; Sokoloff, 1966) and the song variables obtained after performing a principal component analysis (PCA; see below). To this end, we measured wing size of each male using landmark-based geometric morphometrics [with the exception of one male from PMU and one from VEF whose wings broke during dissection (see Table 1 for population
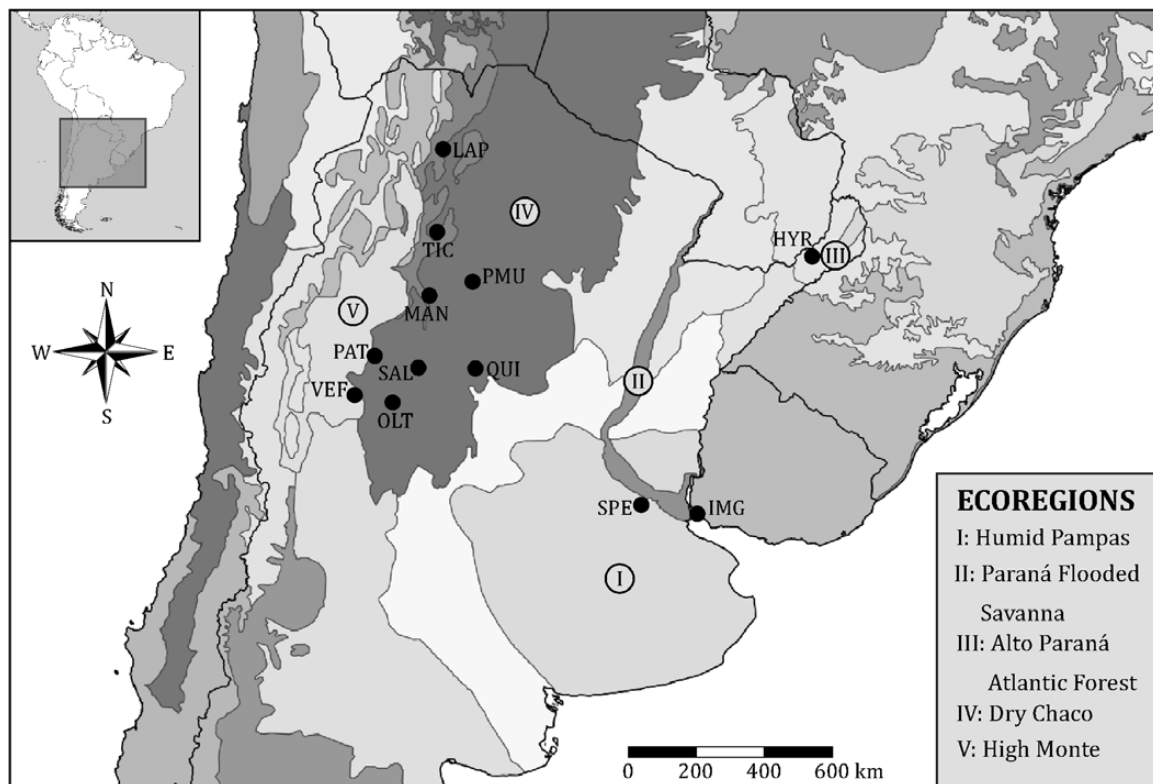

Figure 1. Map of north–central Argentina, showing localities sampled for this study.

Table 1. List of sampling localities along with geographical coordinates, available cactus hosts (C: columnar, P: prickly pears) and the presence of other *D. buzzatii* cluster species

Population	Acronym	Lat. (S)	Long. (W)	Cactus host	Sympatric *D. buzzatii* cluster species
Manantial	MAN	28°07′57″	65°28′31″	P	—
Hipólito Yrigoyen	HYR	27°05′06″	55°17′06″	P	—
Los Lapachos	LAP	24°26′11″	65°04′57″	P-C	—
Isla Martín García	IMG	34°12′49″	58°16′02″	P-C	*D. antonietae*
Olta	OLT	30°36′22″	66°16′20″	P	—
Patquía	PAT	30°02′52″	66°52′42″	P-C	—
Villa Quilino	QUI	30°12′16″	64°28′ 30″	P	—
Salar	SAL	30°09′17″′	65°52′10″′	P-C	*D. koepferae*
San Pedro	SPE	33°39′59″	59°40′46″	P	—
Pampa Muyoj	PMU	27°54′21″	64°26′32″	P	—
Ticucho	TIC	26°34′34″	65°14′24″	P-C	—
Valle Fértil	VFE	30°41′26″	67°29′45″	P-C	*D. koepferae*

codes)]. We scored ten landmarks following Soto *et al.* (2008) and the centroid size (CS) was used as estimator of overall wing size. For correlation analysis, we calculate the mean wing size and the mean of each song variable for each population.

SONG MEASUREMENTS AND ANALYSIS

Courtship songs of 5-day-old males (one male per isofemale line established) were recorded using a SONY ICD-SX712 recorder (for details see Iglesias & Hasson, 2017). We measured four song parameters that characterize both the primary (a) and secondary (b) songs of *D. buzzatii*: the volume at which males emitted each song (Va and Vb), the duration of each song in a burst (Da and Db), the carrier frequency of pulses (CFa and CFb) and the interpulse interval (IPIa and IPIb). Given that primary song has a bimodal distribution of IPIs ('doublets pulses'; see Iglesias & Hasson, 2017: fig. 6), we divided the IPIs of the primary song into short (IPIaS) and long (IPIaL). A mean of five measurements of each parameter per song was used in statistical analysis. All song measurements were estimated in Raven sound analysis software (Raven Pro-1.4 Build 48; Cornell Lab of Ornithology, Bioacoustics Research Program). Pearson correlations were calculated between pairs of all parameters of the primary and secondary songs. For highly correlated parameters ($r \geq 0.80$), only one variable was included in subsequent analyses. As these traits have different units of measurement, we centred and scaled all variables. We then performed a principal component analysis (PCA) to reduce the number of dimensions. The resulting principal components (PCs) with an eigenvalue above 1 were used in subsequent analyses. To interpret the contributions of original variables to

each PC we used the criterion suggested by Mardia *et al.* (1982). Variables with correlations above 0.7 times the largest correlation in an eigenvector were considered to contribute significantly.

Differences in courtship song among populations were evaluated by means of a MANOVA with PCs as dependent variables (see Results for details). To determine which song PCs were driving the patterns revealed by the MANOVA, we ran post hoc ANOVAs for each PC.

PCA was performed using Statistica v.8 (Statsoft Inc., 2007). The remaining analyses were conducted in R v.3.2.5 (R Development Core Team, 2015) using the function Adonis of the Vegan package, which performs an ANOVA using distance matrices and permutations (Oksanen *et al.*, 2013).

INTERPOPULATION GENETIC DIVERGENCE

To estimate levels of genetic divergence between *D. buzzatii* populations, we amplified nine polymorphic microsatellite loci for eight to 15 individuals per population (one fly per isofemale line). We also obtained a fragment of the mitochondrial protein coding gene cytochrome *c* oxidase subunit I (*COI*) for a subset of seven to nine flies per population (one fly per isofemale line).

DNA was extracted using the Puregene Kit (Gentra) following the manufacturer's protocol.

Microsatellite genotyping and analysis

Multilocus genotypes were generated using nine, dinucleotide repeated, microsatellite loci (B3, B54, B63, B65, B65, K60, K72, K75 and K76). Forward primers were tagged at the 5′ end with four different

fluorescent dyes and amplified in two multiplex polymerase chain reaction (PCR) sets using Platinum Multiplex PCR Master Mix (Applied Biosystems). PCR cycling conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 40 s, 58 °C for 30 s and 72 °C for 40 s; and a final extension of 72 °C for 4 min. PCR products were resolved on an ABI 3130XL Genetic Analyser (Applied Biosystems) and sized using Peak Scanner TM software v.1.0 (Applied Biosystems) and GeneMapper software v.3.7 (Applied Biosystems). Primer sequences of the nine microsatellite loci employed in the study are presented in the Supporting Information (Table S1).

All markers have been successfully tested for quality control using wild-collected males (P. Lipko & E. Hasson unpublished; Hurtado *et al.*, 2013). Only two loci (B3 and K72) were in linkage disequilibrium (LD); therefore, we excluded B3 from further analyses. LD between loci were tested using GENEPOP v.4.0 (Rousset, 2008).

The particular method for isofemale lines implemented in the software Microsatellite Analyser v.4.05 (MSA; Dieringer & Schlötterer, 2003; MSA documentation accessible at http://i122server. vu-wien.ac.at/MSA/MSA_download.html) was used to estimate measures of population genetic diversity and divergence. Therefore, the expected heterozygosity (He) and allelic diversity measures were calculated by taking the average of 200 resampled datasets in which one of the two alleles at each locus is randomly discarded (Dieringer & Schlötterer, 2003). MSA with the isofemale line method was also used to calculate pairwise estimates of F_{ST} between populations, and levels of significance were determined by permutation of genotypes 130 000 times among populations (MSA documentation accessible at http://i122server. vu-wien.ac.at/MSA/MSA_download.html); *P*-values were adjusted for multiple testing using Bonferroni correction.

Mitochondrial COI *amplification and analysis*

A 711-bp fragment of the mitochondrial gene *COI* was amplified using the primer C1-N-2191 and a modification of the primer TY-J-1460 (House & Simmons, 2003). Primer sequences are presented in Table S1.

PCRs were performed using GoTaq(R) Green Master Mix (Promega). The PCR programme included an initial denaturing step of 3 min at 94 °C, followed by 35 cycles of amplification (94 °C for 40 s, 48 °C for 40 s and 72 °C for 45 s), with a final extension step at 72 °C for 8 min. Amplified samples were cleaned using 10 U of exonuclease and 1 U of alkaline phosphatase per reaction. PCR products were sequenced in both directions on an ABI 3730 automated sequencer (Macrogen Inc., Korea). Sequences have been deposited in GenBank (accession numbers MH612270–MH612367). Sequences were edited

using Sequencher v.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA), to check for base-calling errors, and BioEdit (Hall, 1999), and were aligned using ClustalW (Thompson *et al.*, 1994).

We employed DNASP v.4.5.3 (Rozas *et al.*, 2003) to obtain the standard measures of genetic diversity and to calculate Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). Both statistics are sensitive to departures from selective neutrality and changes in population size such as expansions or bottlenecks (Tajima, 1996; Fu, 1997). Thus, significant negative values of D and Fs are expected after a population expansion or a selective sweep, whereas positive values are expected under balancing selection or recent bottlenecks. Pairwise estimates of F_{ST} (Weir & Cockerham, 1984) were obtained with ARLEQUIN v.3.5 (Excoffier *et al.*, 2005). A haplotype network was constructed using the software PopART (Leigh & Bryant, 2015) to illustrate the relationships among haplotypes, haplotype frequency and geographical representation.

PATTERNS OF SONG VARIATION BASED ON THE Q_{ST}–F_{ST} APPROACH

To investigate the evolutionary processes driving courtship song variation, we compared quantitative genetic divergence (Q_{ST}) to divergence in putatively neutral molecular markers (F_{ST}). We estimated Q_{ST} using the formula:

$$Q_{ST} = \sigma_B^2 \, / \, (\sigma_B^2 + 2\sigma_W^2)$$

where σ_B^2 and σ_W^2 are the among- and within-population variance, respectively (Leinonen *et al.*, 2013). Pairwise Q_{ST} values were calculated for each PC separately. Variance components for Q_{ST} estimation were obtained using one-way ANOVAs as the dependent variable and pairwise population combinations as the independent variable using the *lme4* package in R v.3.2.5 (R Development Core Team, 2015). Bias in the estimation of Q_{ST} using REML was appreciable only at high values of Q_{ST} (> 0.7; O'Hara & Merilä, 2005), which is not the case for this study.

Inference regarding significant differences between F_{ST} and Q_{ST} was based on the posterior weighted mean and 95% highest density intervals using a Bayesian bootstrap method with 10 000 posterior resamples within the R package 'bayesboot' (Bååth, 2016).

RESULTS

ALLOMETRY IN SONG VARIABLES

Wing size differed significantly across populations (ANOVA: $F11,139 = 2.79$; $P < 0.01$). However, mean wing sizes did not correlate significantly with any of the song variables ($r \leq 0.23$ and $P \geq 0.06$, for all tests).

POPULATION DIVERGENCE IN COURTSHIP SONG

The volume of primary (Va) and secondary (Vb) song were the only highly correlated parameters found (r = 0.82). Therefore, we only used Vb in subsequent data analysis. PCA of eight measures of courtship song returned three components (PCs) with eigenvalues greater than 1, which collectively explained 54% of song variance. PC1 explained 22% of the total song variation, and was loaded positively by the long IPI of the primary song (IPIaL) and the IPI of the secondary song (IPIb); PC2 explained 17% of the total variation, and was loaded positively by the short IPI of the primary song (IPIaS) and the spectral parameters of both songs (FDa and FDb); PC3 explained 15% of the total variation, and was loaded positively by the duration of both types of songs (Da and Db).

As wing centroid size was not a significant predictor of song PCs, differences in courtship song among populations were evaluated by means of a MANOVA with PCs as dependent variables. MANOVA showed that courtship song differs significantly among populations ($F_{11,141}$ = 2.15, $P < 0.001$). Post-hoc tests revealed a significant population effect on PC2 (ANOVA: $F_{11,138}$ = 2.96; $P < 0.01$) and PC3 (ANOVA: $F_{11,138}$ = 3.28; $P < 0.001$), but not on PC1. A scatterplot showing population clustering patterns based on the two PCs showing geographical variation (PC2 and PC3) is presented in Figure 2.

Population divergence at microsatellite loci

The number of alleles in the microsatellite loci ranged from 5.87 to 7.44 per population, with an average of 7.58 alleles per locus (see Table S2 for summaries of microsatellite diversity). F_{ST} values did not differ significantly from zero in all pairwise population combinations (see Table S3 for pairwise F_{ST} estimates).

Mitochondrial DNA diversity and population divergence

The analysis of a 723-bp sequence fragment of *COI* from 98 individuals recovered 21 haplotypes (see Table S2 for summaries of mtDNA diversity). The constructed haplotype network revealed very low levels of differentiation among haplotypes; most of them differed by only a single mutational step from the common haplotype (Fig. 3). With the exception of populations MAN and QUI, unique haplotypes were found in all populations. However, one haplotype contained the majority of individuals (Fig. 3).

AMOVA showed that most variation (> 98%) was harboured within populations. Accordingly, F_{ST} values did not differ significantly from zero in all pairwise population combinations (see Table S3 for pairwise F_{ST} estimates).

The distributions of Tajima's D and Fu's Fs were significantly skewed towards negative values (–2.53, $P < 0.01$ and –31.49, $P < 0.001$, respectively).

EVOLUTIONARY FORCES UNDERLYING COURTSHIP SONG VARIATION

Highest density intervals (HDIs) for the posterior mean pairwise Q_{ST} values describing PC1 overlapped with those for the posterior mean pairwise F_{ST} values estimated either from microsatellites or from *COI* (i.e. $Q_{ST} = F_{ST}$; Fig. 4). Thus, we cannot reject the null hypothesis of neutral evolution for PC1. However, HDI for the posterior mean pairwise Q_{ST} values for PC2 and PC3 were greater than HDI for the posterior mean pairwise F_{ST} values estimated for both microsatellites and *COI* (i.e. $Q_{ST} > F_{ST}$; Fig. 4). These results suggest that directional selection would be operating upon both PC2 and PC3 variables (i.e. $F_{ST} < Q_{ST}$).

DISCUSSION

In this study we have documented divergence in courtship song among populations of *D. buzzatii* in the face of genetic homogeneity and no population structure. The species showed low mitochondrial nucleotide diversity and high haplotype diversity. This, together with haplotypes distributed in a star-like network and significant negative values of D and Fs statistics, is consistent with a recent range expansion possibly associated with post-glacial colonization and the increase in intensive cultivation of commercial prickly pears in north-western Argentina (Manfrin & Sene, 2006; Ervin, 2012). Despite the higher mutation
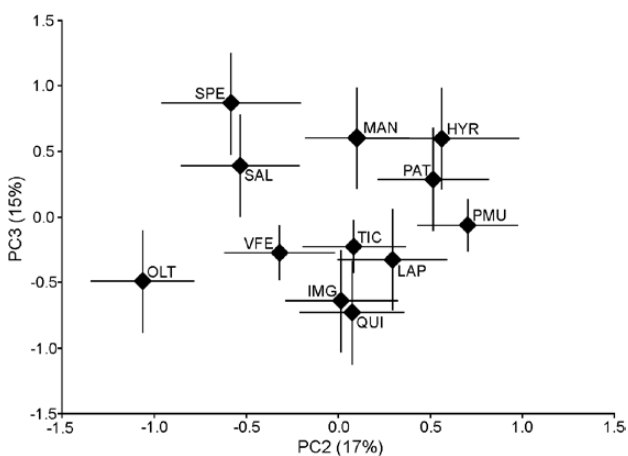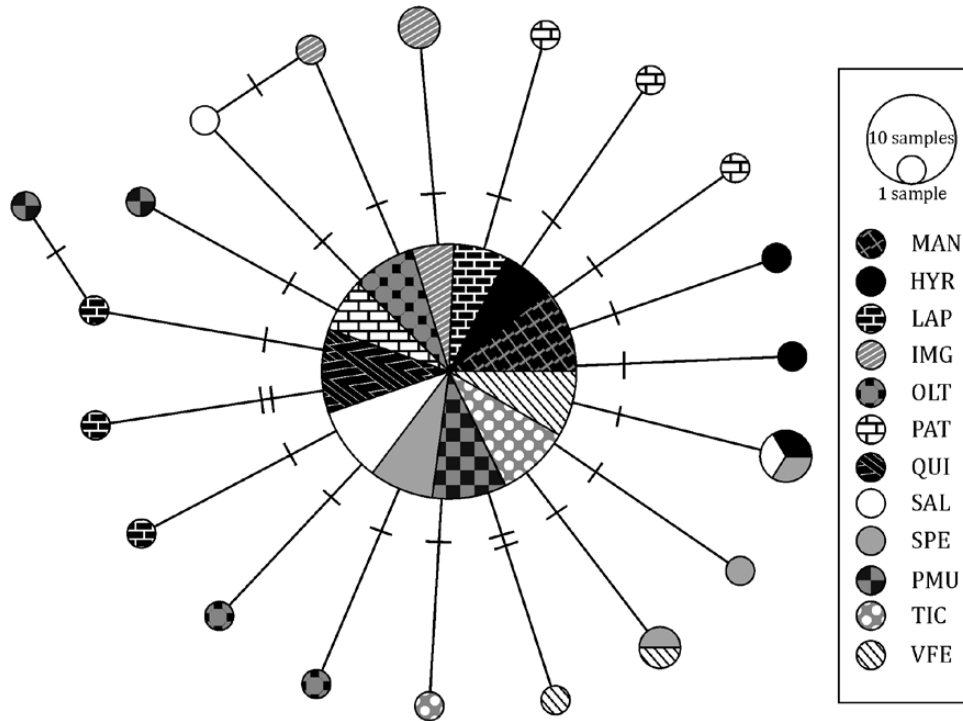

Figure 2. Scatterplot of mean song scores (and standard errors) showing group separation in courtship song using the two PCs (PC2 and PC3), which showed among-population variation.

Figure 3. Network of mtDNA haplotypes. Each circle represents a single haplotype and its size is proportional to the number of individuals bearing that particular haplotype. Each short line crossing the branches represents a single mutational step. Different patterns and shades of grey refer to different populations (see Table 1).
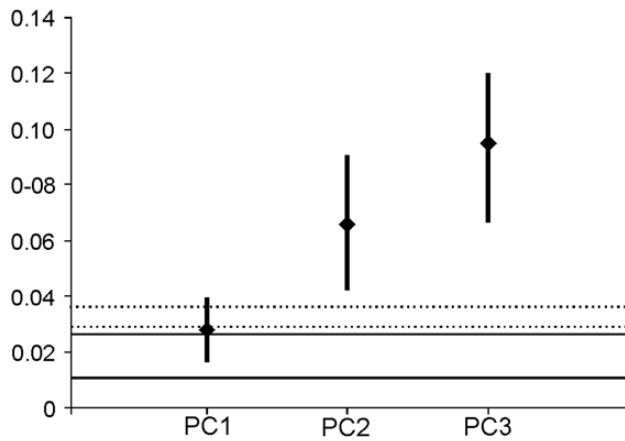

Figure 4. Posterior weighted mean and 95% highest density intervals, obtained by Bayesian bootstrapping, for pairwise Q_{ST} estimates for PCs describing song variation. Horizontal dashed lines and horizontal solid lines indicate upper and lower 95% highest density limits for pairwise F_{ST} values estimated from microsatellites and *COI*, respectively.

rates of microsatellites compared to mitochondrial DNA, we did not find evidence for population genetic structure. These results are consistent with previous studies, using different molecular markers, showing

evidence for population expansion and genetic homogeneity in *D. buzzatii* (Rossi *et al.*, 1996; Rodriguez *et al.*, 2000; Gómez & Hasson, 2003; Laayouni *et al.*, 2003; Piccinali *et al.*, 2007). Nevertheless, we found among-population differentiation in courtship song involving spectral parameters, CFa and CFb, and the temporal parameters IPIaS, Da and Db. We have used, for the first time in *Drosophila*, the Q_{ST}–F_{ST} approach to investigate the relative roles of selective and neutral processes in the evolution of courtship song. Our results are consistent with a role for directional selection causing song divergence among populations. Because local adaptation in the face of high gene flow depends on the strength of the local selective force, our results suggest that strong selective pressures are driving song differentiation (Muir *et al.*, 2014).

ON THE ROLE OF SELECTIVE AND NEUTRAL PROCESSES IN SONG GEOGRAPHICAL DIFFERENTIATION

Geographical song variation in many *Drosophila* species is thought to be driven by rapid and strong evolutionary forces. However, previous studies in *Drosophila* have focused mainly on isolated populations and lacked an empirical approach to differentiate between genetic drift and selection as drivers of song variation. Among cactophilic *Drosophila*, geographical

© 2018 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2018, **XX**, 1–12

variation in song IPI and burst duration has been documented in *D. mojavensis* (Etges *et al.*, 2006), and song IPI and CF variation have been documented in *D. meridionalis* (Costa *et al.*, 2000). Because these species display spatially restricted gene flow (Zouros & d'Entremont, 1974; Markow, 1991; Costa *et al.*, 2000; Etges *et al.*, 2006), caution must be exerted in the interpretation of the evolutionary forces causing song divergence. A major obstacle is the lack of information on neutral genetic differentiation among populations (i.e. F_{ST}; Wilkins *et al.*, 2013). In this sense, Wojcieszek & Simmons (2012), studying genital variation among isolated populations of the millipede *Antichiropus variabilis*, found that although genital morphology was highly divergent among populations, genital divergence was significantly lower than that experienced by neutral genetic markers (i.e. $Q_{ST} < F_{ST}$). In addition, a recent study showed that although substantial evidence supports the existence of female preferences for three sexual traits (including song) in the field cricket *Teleogryllus oceanicus*, genetic drift appears to have played a dominant role in generating population differences (Pascoal *et al.*, 2017). In this context, the Q_{ST}–F_{ST} technique can be a valuable approach to investigate the relative roles of selection and neutral processes. Among non-cactophilic *Drosophila*, the evolution of courtship song has been most thoroughly studied in *D. montana* (Hoikkala & Mazzi, 2009). Song IPI and CF, and at least female preferences for CF, have been found to vary among genetically differentiated populations (Klappert *et al.*, 2007; Routtu *et al.*, 2007). Although some evidence suggests that random genetic drift would not be sufficient to explain the pattern of song variation as a whole (Routtu *et al.*, 2007), the lack of a more focused approach, like the Q_{ST}–F_{ST} approach, led to the same uncertainties about what evolutionary processes have actually occurred and on what song parameters.

Evidence of local adaptation in the face of high gene flow is scarce. Nonetheless, Muir *et al.* (2014) reported this scenario for several larval traits in the common frog *Rana temporaria* using the Q_{ST}–F_{ST} approach.

ON THE ROLE OF COURTSHIP SONG PARAMETERS

A previous study demonstrated that song is part of the mate recognition system in *D. buzzatii* (Iglesias & Hasson, 2017). Accordingly, we expected some parameters to be under stabilizing selection, homogenizing variation among populations (i.e. $Q_{ST} < F_{ST}$; Whitlock, 2008; Hasson *et al.*, 2013; Leinonen *et al.*, 2013). The lack of significant among-population variation for PC1 is consistent with the little population differentiation expected for mate recognition traits (McPeek *et al.*, 2008, 2009). However, none of the Q_{ST} values was significantly lower than the F_{ST} values. In this

sense, the extremely low F_{ST} values found may preclude the detection of stabilizing selection. Weak stabilizing selection due to a wide female preference window (Butlin, 1993; also see Ryan & Rand, 1993; Castellano & Cermelli, 2006) could explain the high levels of genetic variation found within populations for PC1. The fact that PC1 is loaded by IPIaL and IPIb is consistent with the importance of IPI in mate recognition that has been demonstrated in several species of *Drosophila* (reviewed by Tomaru & Yamada, 2011). Also, the combination of IPIs from both songs in PC1 is in line with the idea that females of the *D. buzzatii* cluster may use more than one song parameter in mate recognition (Oliveira *et al.*, 2013). Oliveira *et al.* (2013) found significant overlap among species when a single parameter was considered independently of the others. Previous studies on experimental evolution, investigating the consequences of sexual selection acting on male genital morphology in the dung beetle *Onthophagus taurus*, found no significant evolutionary divergence in response to sexual selection in traits subject to patterns of non-linear selection (characteristic of stabilizing and disruptive selection) but found significant evolutionary divergence in traits subject to linear selection (Simmons *et al.*, 2009). In addition, in line with our results, the authors did not find significant divergence for the first PC, which accounted for 35% of the variation on genital morphology. All in all, we cannot rule out the existence of high levels of genetic variation for female mean preference within populations, which could mimic a scenario of stabilizing selection. Therefore, playback experiments are needed to confirm the role of IPIaL and IPIb in mate recognition.

Given the slow rate of evolution of mate recognition signals, geographical variation in signals subject to stabilizing selection is more likely to occur in species with some degree of genetic structuration rather than in species with high levels of gene flow (see exmples in Wojcieszek & Simmons, 2012; Wong *et al.*, 2004). Nevertheless, geographical variation in mate recognition signals could also be expected as a result of character displacement (Coyne & Orr, 2004). In such a case, it is expected that populations of *D. buzzatii* living in sympatry with closely related species will be acoustically more differentiated than allopatric ones. Only three out of 12 populations sampled were sympatric (Table 1). However, the pattern of among-population variation observed is not consistent with this hypothesis (Fig. 2; Higgie *et al.*, 2000).

On the other hand, we found evidence for divergent selection on PC2 and PC3 variables, involving the short IPI of primary song (IPIaS), the spectral parameters (CFa and CFb) and the duration of both songs (Da and Db). These results are consistent with the idea of sexually selected parameters experiencing continuous directional selection within populations,

leading to rapid divergence among populations (i.e. $Q_{ST} > F_{ST}$). Substantial variation in female preference for CF was documented between geographically and phylogenetically distinct populations of *D. montana* (Klappert *et al.*, 2007). Females of this species do not exert any non-linear selection on CF and the level of responsiveness and the shape (slope) of the preference function varied significantly among populations. In addition, female preference for longer pulse trains has been documented in a wild type stock of *D. melanogaster* (Talyn & Dowse, 2004).

ON THE ROLE OF SEXUAL SELECTION IN SONG ELABORATION

Ewing & Miyan (1986) suggested that the most elaborated features of courtship song in the *D. repleta* species group are doublet pulses and composite bursts. Accordingly, sexual selection may be operating on these song features as was proposed by the traditional model of sexual selection (Darwin, 1871). Our results suggest that different selective pressures are acting simultaneously on both IPIs of primary song. Thus, putatively directional selection acting on IPIaS is likely to have contributed to the origin and maintenance of the doublet pulses. However, the role of sexual selection on composite burst is more difficult to interpret. On the one hand, our results suggest that IPIaL of primary song and IPIb of secondary song (PC1) are likely to be implicated in mate recognition rather than in mate choice. From this perspective, composite bursts could be associated with the way in which females process information allowing simultaneous assessment of both parameters (Castellano & Cermelli, 2006). On the other hand, our results also suggest that directional selection is acting on the duration of both songs (Da and Db), a parameter that has been traditionally associated with singing effort (Prestwich & Walker, 1981; Kavanagh, 1987; Prestwich, 1994; Hunt *et al.*, 2004). Due to the energetic costs of increased signal production, it has been proposed that such signals may convey information about the phenotypic and/or genetic quality of males (Hunt *et al.*, 2004; Bentsen *et al.*, 2006). Singing in *Drosophila* requires energetically expensive muscle contractions (reviewed by Bennet-Clark, 1998) and sex-specific evolution of large sound-producing muscles has been documented in *D. melanogaster* (Shirangi *et al.*, 2013). It is striking that secondary song of *D. buzzatii* tends to be shorter than primary song and that it only occurs in composite bursts (Oliveira *et al.*, 2013; Iglesias & Hasson, 2017). From this perspective, one might ask whether secondary song has a higher energetic cost associated and the evolution of composite bursts could be the result of selective pressures for longer bursts. Playback experiments using synthetic songs are needed to understand the relative role of simple and composite bursts in mating success.

CONCLUSIONS

As far as we know, it is the first study showing courtship song divergence despite the lack of neutral genetic differentiation in *Drosophila*. The novelty of our study lies in the combination of neutral molecular markers and quantitative genetic data obtained from a common-garden experiment to differentiate between selective and neutral processes causing song divergence. Our results suggest that continuous directional selection has promoted divergence in a signal involved in mate recognition, which supports the idea that evolution of courtship song in nature is highly complex. Although our results need to be validated in playback experiments, we showed that Q_{ST}–F_{ST} comparisons are a useful initial approach to unravel the role of the multiple components of courtship songs.

ACKNOWLEDGEMENTS

REFERENCES

Akre KL, Ryan MJ. 2010. Proximity-dependent response to variably complex mating signals in túngara frogs (*Physalaemus pustulosus*). *Ethology* **116:** 1138–1145.

Amorim MCP. 2006. Diversity of sound production in fish. *Communication in Fishes* **1:** 71–104.

Andersson MB. 1994. *Sexual selection*. Princeton: Princeton University Press.

Arbuthnott D, Elliot MG, McPeek MA, Crespi BJ. 2010. Divergent patterns of diversification in courtship and genitalic characters of *Timema* walking-sticks. *Journal of Evolutionary Biology* **23:** 1399–1411.

Arnqvist G. 1997. The evolution of animal genitalia: distinguishing between hypotheses by single species studies. *Biological Journal of the Linnean Society* **60:** 365–379.

Bååth R. 2016. *bayesboot: an implementation of Rubin's (1981) Bayesian Bootstrap*. R package version 0.2.2. Available at: https://github.com/rasmusab/bayesboot

Bennet-Clark HC. 1998. Size and scale effects as constraints in insect sound communication. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **353:** 407–419.

Bentsen CL, Hunt J, Jennions MD, Brooks R. 2006. Complex multivariate sexual selection on male acoustic signaling in a wild population of *Teleogryllus commodus*. *The American Naturalist* **167:** E102–E116.

Bevier CR, Gomes FR, Navas CA. 2008. Variation in call structure and calling behavior in treefrogs of the genus *Scinax*. *South American Journal of Herpetology* **3:** 196–206.

Blair WF. 1955. Mating call and stage of speciation in the *Microhyla olivacea–M. carolinensis* complex. *Evolution* **9:**469–480.

Butlin RK. 1993. The variability of mating signals and preferences in the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). *Journal of Insect Behavior* **6:** 125–140.

Castellano S, Cermelli P. 2006. Reconciling sexual selection to species recognition: a process-based model of mating decision. *Journal of Theoretical Biology* **242:** 529–538.

Catchpole CK, Slater PJB. 2003. *Bird song: biological themes and variations*. New York: Cambridge University Press.

Cocroft RB, De Luca P. 2006. Size-frequency relationships in insect vibratory signals. In: Drosopoulos S, Claridge MF, eds. *Insect sounds and communication: physiology, behavior, ecology and evolution*. Boca Raton: CRC Press, 99–110.

Collins SA, de Kort SR, Pérez-Tris J, Tellería JL. 2009. Migration strategy and divergent sexual selection on bird song. *Proceedings of the Royal Society of London B: Biological Sciences* **276:** 585–590.

Costa CTA, Kuhn GCS, Sene FM. 2000. Low courtship song variation in South and Southeastern Brazilian populations of *Drosophila meridionalis* (Diptera, Drosophilidae). *Revista Brasileira de Biologia* **60:**53–61.

Coyne JA, Orr HA. 2004. *Speciation*. Sunderland: Sinuaer Associates.

Darwin C. 1859. *On the origin of the species by natural selection*. London: John Murray.

Darwin C. 1871. *The descent of man and selection in relation to sex*. London: John Murray.

Dieringer D, Schlötterer C. 2003. Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Resources* **3:** 167–169.

Dobzhansky T. 1940. Speciation as a stage in evolutionary divergence. *The American Naturalist* **74:** 312–321.

Drosopoulos S, Claridge MF. 2005. *Insect sounds and communication: physiology, behaviour, ecology, and evolution*. Boca Raton: CRC Press.

Ervin GN. 2012. Indian fig cactus (*Opuntia ficus-indica* (L.) Miller) in the Americas: an uncertain history. *Haseltonia* **17:** 70–81.

Etges WJ, Over KF, De Oliveira CC, Ritchie MG. 2006. Inheritance of courtship song variation among geographically isolated populations of *Drosophila mojavensis*. *Animal Behaviour* **71:**1205–1214.

Ewing AW, Miyan JA. 1986. Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. *Animal Behaviour* **34:** 421–429.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* **1:** 47–50.

Ferreira M, Ferguson JWH. 2002. Geographic variation in the calling song of the field cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae) and its relevance to mate recognition and mate choice. *Journal of Zoology* **257:** 163–170.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147:** 915–925.

Gerhardt HC. 1991. Female mate choice in treefrogs: static and dynamic acoustic criteria. *Animal Behaviour* **42:** 615–635.

Gerhardt HC, Brooks R. 2009. Experimental analysis of multivariate female choice in gray treefrogs (*Hyla versicolor*): evidence for directional and stabilizing selection. *Evolution* **63:** 2504–2512.

Gómez GA, Hasson E. 2003. Transpecific polymorphisms in an inversion linked esterase locus in *Drosophila buzzatii*. *Molecular Biology and Evolution* **20:** 410–423.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hasson ER, Fanara JJ, Frankel N. 2013. Selection and complex multigene traits. In: eLS. Chichester: John Wiley & Sons, Ltd, 1–13. doi:10.1002/9780470015902.a0002295

Higgie M, Chenoweth S, Blows MW. 2000. Natural selection and the reinforcement of mate recognition. *Science* **290:** 519–521.

Hoikkala A, Mazzi D. 2009. Evolution of complex acoustic signals in *Drosophila* species. In: Kim YK, ed. *Handbook of behavior genetics*. Berlin: Springer-Verlag, 187–196.

Hosken DJ, Stockley P. 2004. Sexual selection and genital evolution. *Trends in Ecology & Evolution* **19:** 87–93.

House CM, Simmons LW. 2003. Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. *Proceedings of the Royal Society of London B: Biological Sciences* **270:** 447–455.

Howard DJ. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In: Harrison RG, ed. *Hybrid zones and the evolutionary process*. Ithaca: Cornell University Press, 46–69.

Hunt J, Brooks R, Jennions MD, Smith MJ, Bentsen CL, Bussière LF. 2004. High-quality male field crickets invest heavily in sexual display but die young. *Nature* **432:** 1024–1027.

Hurtado J, Iglesias PP, Lipko P, Hasson E. 2013. Multiple paternity and sperm competition in the sibling species *Drosophila buzzatii* and *Drosophila koepferae*. *Molecular Ecology* **22:** 5016–5026.

Iglesias PP, Hasson E. 2017. The role of courtship song in female mate choice in South American cactophilic *Drosophila*. *PloS One* **12:** e0176119.

Kavanagh MW. 1987. The efficiency of sound production in two cricket species, *Gryllotalpa australis* and *Teleogryllus commodus* (Orthoptera: Grylloidea). *Journal of Experimental Biology* **130:** 107–119.

Klappert K, Mazzi D, Hoikkala A, Ritchie MG. 2007. Male courtship song and female preference variation between phylogeographically distinct populations of *Drosophila montana*. *Evolution* **61:** 1481–1488.

Laayouni H, Hasson E, Santos M, Fontdevila A. 2003. The evolutionary history of *Drosophila buzzatii*. XXXV. Inversion polymorphism and nucleotide variability in different regions of the second chromosome. *Molecular Biology and Evolution* **20:** 931–944.

Leigh JW, Bryant D. 2015. popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6:** 1110–1116.

Leinonen T, McCairns RJ, O'Hara RB, Merilä J. 2013. Q(ST)-F(ST) comparisons: evolutionary and ecological insights from genomic heterogeneity. *Nature Reviews. Genetics* **14:** 179–190.

Manfrin MH, Sene FM. 2006. Cactophilic *Drosophila* in South America: a model for evolutionary studies. *Genetica* **126:** 57–75.

Mardia KV, Kent JT, Bibby JM. 1982. *Multivariate analysis*. New York: Academic Press.

Markow TA. 1991. Sexual isolation among populations of *Drosophila mojavensis*. *Evolution* **45:** 1525–1529.

Mather K. 1973. *Genetical structure of populations*. London: Chapman and Hall.

McPeek MA, Shen L, Farid H. 2009. The correlated evolution of three-dimensional reproductive structures between male and female damselflies. *Evolution* **63:** 73–83.

McPeek MA, Shen L, Torrey JZ, Farid H. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. *The American Naturalist* **171:** E158–E178.

McPeek MA, Symes LB, Zong DM, McPeek CL. 2011. Species recognition and patterns of population variation in the reproductive structures of a damselfly genus. *Evolution* **65:** 419–428.

Muir AP, Biek R, Thomas R, Mable BK. 2014. Local adaptation with high gene flow: temperature parameters drive adaptation to altitude in the common frog (*Rana temporaria*). *Molecular Ecology* **23:** 561–574.

Oh KP, Shaw KL. 2013. Multivariate sexual selection in a rapidly evolving speciation phenotype. *Proceedings of the Royal Society of London B: Biological Sciences* **280:** 20130482.

O'Hara RB, Merilä J. 2005. Bias and precision in QST estimates: problems and some solutions. *Genetics* **171:** 1331–1339.

Okanoya K. 2015. Evolution of song complexity in Bengalese finches could mirror the emergence of human language. *Journal of Ornithology* **156:**65–72.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Simpson GL, Solymos P, Stevens MHS,

Szoecs E, Wagner H. 2013. *Package 'vegan'*. R package version 1.17–14. Oulu, Finland: University of Oulu. Available at: http://CRAN.R-project.org/package=vegan

Oliveira CC, Manfrin MH, Sene FM, Etges WJ. 2013. Evolution of male courtship songs in the *Drosophila buzzatii* species cluster. In: Michalak P, ed. *Speciation: natural processes, genetics and biodiversity*. New York: Nova Science Publishers, 137–164.

Pascoal S, Mendrok M, Wilson AJ, Hunt J, Bailey NW. 2017. Sexual selection and population divergence II. Divergence in different sexual traits and signal modalities in field crickets (*Teleogryllus oceanicus*). *Evolution* **71:** 1614–1626.

Patterson JT, Wheeler MR. 1942. Description of a new species of the subgenera *Hirtodrosophila* and *Drosophila*. *University of Texas Publications* **4213:** 62–109.

Paterson HEH, Shane FM. 1993. *Evolution and the recognition concept of species: collected writings*. Baltimore: Johns Hopkins University Press.

Piccinali RV, Mascord LJ, Barker JS, Oakeshott JG, Hasson E. 2007. Molecular population genetics of the alpha-esterase5 gene locus in original and colonized populations of *Drosophila buzzatii* and its sibling *Drosophila koepferae*. *Journal of Molecular Evolution* **64:** 158–170.

Podos J, Patek SN. 2015. Acoustic signal evolution: biomechanics, size, and performance. In: Irschick DJ, Briffa M, Podos J, eds. *Animal signaling and function: an integrative approach*. Hoboken: John Wiley & Sons, 175–203.

Prestwich KN. 1994. The energetics of acoustic signaling in anurans and insects. *American Zoologist* **34:** 625–643.

Prestwich KN, Walker TJ. 1981. Energetics of singing in crickets: effect of temperature in three trilling species (Orthoptera: Gryllidae). *Journal of Comparative Physiology* **143:**199–212.

R Development Core Team. 2015. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Robertson FW. 1962. Changing the relative size of the body parts of *Drosophila* by selection. *Genetics Research* **3:** 169–180.

Rodriguez C, Piccinali R, Levy E, Hasson E. 2000. Contrasting population genetic structures using allozymes and the inversion polymorphism in *Drosophila buzzatii*. *Journal of Evolutionary Biology* **13:** 976–984.

Rossi MS, Barrio E, Latorre A, Quezada-Díaz JE, Hasson E, Moya A, Fontdevila A. 1996. The evolutionary history of *Drosophila buzzatii*. XXX. Mitochondrial DNA polymorphism in original and colonizing populations. *Molecular Biology and Evolution* **13:** 314–323.

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8:** 103–106.

Routtu J, Mazzi D, Van der Linde K, Mirol P, Butlin RK, Hoikkala A. 2007. The extent of variation in male song, wing and genital characters among allopatric *Drosophila montana* populations. *Journal of Evolutionary Biology* **20:** 1591–1601.

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19:** 2496–2497.

Ryan MJ, Rand AS. 1993. Species recognition and sexual selection as a unitary problem in animal communication. *Evolution* **47:** 647–657.

Shaw KL, Herlihy DP. 2000. Acoustic preference functions and song variability in the Hawaiian cricket *Laupala cerasina*. *Proceedings of the Royal Society of London B: Biological Sciences* **267:**577–584.

Shirangi TR, Stern DL, Truman JW. 2013. Motor control of *Drosophila* courtship song. *Cell Reports* **5:** 678–686.

Simmons LW, House CM, Hunt J, García-González F. 2009. Evolutionary response to sexual selection in male genital morphology. *Current Biology* **19:** 1442–1446.

Sokoloff A. 1966. Morphological variation in natural and experimental populations of *Drosophila pseudoobscura* and *Drosophila persimilis*. *Evolution* **20:** 49–71.

Soto IM, Carreira VP, Soto EM, Hasson E. 2008. Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*. *Journal of Evolutionary Biology* **21:** 598–609.

StatSoft. 2007. *STATISTICA version 8*. Tulsa, Oklahoma: StatSoft Inc.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585–595.

Tajima F. 1996. The amount of DNA polymorphism maintained in a finite population when the neutral mutation rate varies among sites. *Genetics* **143:** 1457–1465.

Talyn BC, Dowse HB. 2004. The role of courtship song in sexual selection and species recognition by female *Drosophila melanogaster*. *Animal Behaviour* **68:** 1165–1180.

Tanner JC, Ward JL, Shaw RG, Bee MA. 2017. Multivariate phenotypic selection on a complex sexual signal. *Evolution* **71:** 1742–1754.

Templeton AR. 1979. Once again, why 300 species of Hawaiian *Drosophila*? *Evolution* **33:** 513–517.

Thinh VN, Hallam C, Roos C, Hammerschmidt K. 2011. Concordance between vocal and genetic diversity in crested gibbons. *BMC Evolutionary Biology* **11:** 36.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Tomaru M, Yamada H. 2011. Courtship of *Drosophila*, with a special interest in courtship songs. *Low Temperature Science* **69:** 61–85.

Vilela CR. 1983. Revision of the *Drosophila repleta* species group (Diptera, Drosophilidae). *Revista Brasileira de Entomologia* **27:** 1–114.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38:** 1358–1370.

West-Eberhard MJ. 2003. *Developmental plasticity and evolution*. Oxford: Oxford University Press.

Whitlock MC. 2008. Evolutionary inference from QST. *Molecular Ecology* **17:** 1885–1896.

Wilkins MR, Seddon N, Safran RJ. 2013. Evolutionary divergence in acoustic signals: causes and consequences. *Trends in Ecology & Evolution* **28:** 156–166.

Wojcieszek JM, Simmons LW. 2012. Evidence for stabilizing selection and slow divergent evolution of male genitalia in a millipede (*Antichiropus variabilis*). *Evolution* **66:** 1138–1153.

Wong BB, Keogh JS, Jennions MD. 2004. Mate recognition in a freshwater fish: geographical distance, genetic differentiation, and variation in female preference for local over foreign males. *Journal of Evolutionary Biology* **17:** 701–708.

Zouros E, d'Entremont CJ. 1974. Sexual isolation among populations of *Drosophila mojavensis* race B. *Drosophila Information Service* **51:** 112.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Primer sequences used to obtain molecular data.
Table S2. Mitochondrial and microsatellite diversity statistics.
Table S3. Genetic differentiation among populations estimated by means of F_{ST} values from mtDNA (below diagonal) and microsatellites (above diagonal).
Table S4. Song differentiation among populations estimated by means of Q_{ST} values from PC1 (below diagonal) and PC2 (above diagonal).
Table S5. Song differentiation among populations estimated by means of Q_{ST} values from PC3.