In: Speciation: Natural Processes, Genetics and Biodiversity. 2013. P. Michalak (ed.), pp. 137-164, Nova Science Publishers, Inc., New York.

Chapter

EVOLUTION OF MALE COURTSHIP SONGS IN THE *DROSOPHILA BUZZATII* SPECIES CLUSTER

***Cássia C. Oliveira*[1,2,*], *Maura H. Manfrin*[3], *Fábio de M. Sene*[4] and *William J. Etges*[1]**
[1]Program in Ecology and Evolutionary Biology, Department of Biological Sciences, University of Arkansas,Fayetteville, AR, US
[2]Math and Science Division, Lyon College, Batesville, AR, US
[3]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brazil
[4]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brazil

ABSTRACT

Acoustic signals produced to attract mates before, during, and after courtship are frequently involved with sexual selection, sexual isolation, and reproductive isolation in *Drosophila* spp. and other animals, yet few studies have revealed how courtship songs evolve in a larger phylogenetic context. Therefore, we mapped different acoustic components of courtship songs in the monophyletic *Drosophila buzzatii* species cluster onto an independently derived *period* (*per*) gene + chromosome inversion phylogeny to assess the concordance of courtship song evolution with species divergence. These cactophilic flies are distributed throughout several biomes in southern South America and include the sibling species *D. buzzatii, D. koepferae, D. serido, D. borborema, D. seriema, D. antonietae,* and *D. gouveai*. All seven species produced two song types; primary and secondary pulse songs, except for *D. borborema* and *D. gouveai* that produced no secondary songs. Courtship songs were characterized by analyzing six commonly studied acoustic components including burst duration (BD), carrier frequency (CF), pulse length (PL), pulse number (PN), inter-burst interval (IBI), and inter-pulse interval (IPI). Significant intra- and inter-specific song variation was observed for BD, PN, and IBI, while CF, PL, and IPI varied in a more species-specific manner, albeit with some overlap. Thus, some song components may be better species recognition signals

---

[*] Email: cassia.oliveira@lyon.edu.

than others. Multivariate clustering analyses resolved all species into distinct, non-overlapping groups. Mapping individual song traits (BD, IBI, and IPI) as well composites of these song variables onto our (*per*) gene + chromosome inversion phylogeny revealed no phylogenetic signal when different comparative mapping methods were used. Hence, the evolution of courtship songs in *D. buzzatii* cluster species was uncorrelated with the degree of species divergence. These findings reinforce previous observations that courtship songs evolve rapidly enough to erase any signature of evolutionary affinity between closely related animal species.

INTRODUCTION

In a number of *Drosophila* species, courtship “love” songs have been implicated in promoting sexual isolation between species (Ewing 1989; Ritchie et al. 1999). Despite the potential importance of courtship songs in the speciation process and that songs have been characterized in over 100 *Drosophila* species (Hoikkala 2005), only a few studies have investigated the correlation between song traits and species phylogenetic history (Ewing and Miyan 1986; Gleason and Ritchie 1998; Etges 2002). Comparative studies involving mate signaling cues in closely related species are crucial to unraveling not only which traits are repeatedly involved in the early stages of species formation, but also determining their divergence rates across taxa (Etges 2002; Coyne and Orr 2004). In short, we are interested in identifying key behavioral traits that are responsible for large-scale diversification of species. Thus, we analyzed the evolution of quantitative differences in courtship song traits in the *D. buzzatii* cluster, a group of recently diverged species, in order to assess the concordance of love song evolution in relation to patterns of species divergence in a phylogenetic context.

During courtship, males of most *Drosophila* species produce acoustic signals, courtship love songs, by vibrating their wings in attempts to gain female acceptance and successful copulation (Ewing 1983). Courtship songs are typically species-specific in the majority of *Drosophila* species (Cowling and Burnet 1981; Cobb et al. 1988; Ritchie and Gleason 1995), and so acoustic signaling is thought to allow courting adults to ascertain the appropriateness of attempting to mate with a member of the opposite sex (Ewing 1989; Saarikettu et al. 2005; Mendelson and Shaw 2012), even though these species differences may evolve by sexual selection (Ritchie and Gleason 1995; Ritchie et al. 1998).

Drosophila love songs are typically characterized by low frequency pulses that can be produced individually or in structured bursts. Some species, such as those in the *D. melanogaster* group, have two types of song, pulse song and sine song (Cowling and Burnet 1981). In the *D. repleta* group, two kinds (A and B) of pulse songs have been described where A songs have short inter-pulse intervals (S-IPIs), and B songs have longer inter-pulse intervals (L-IPIs) (Figure 20). However, not all species within the group exhibit both song types, and variation between species is considerable (Ewing and Miyan 1986).

The rate of pulse production measured by the inter-pulse interval or IPI has been shown to be a common mate recognition signal recognized by female *Drosophila*. However, other courtship song traits have been found to be species-specific in *Drosophila*, including burst duration, inter-burst interval, pulse number per burst, sine song, cycle number per pulse, and intra-pulse frequency (Bennet-Clark and Ewing 1969; Kyriacou and Hall 1980; Ritchie and Gleason 1995; Byrne 1999; Yamada et al. 2002; Etges et al. 2006). Females can use one or more love song components when selecting among potential mates (Kyriacou and Hall 1982; Tomaru et al. 1995; Ritchie, et al. 1998). For example, *Drosophila montana* females will not mate with wingless males, implying that courtship song is an obligatory component of courtship in this species (Liimatainen et al. 1992). In *D. melanogaster*, male courtship song is not necessary for mate recognition, since wingless males can copulate, even though the time it takes to achieve copulation is longer than for control males (von Schilcher 1976). Using recorded love songs in playback experiments has shown a role for courtship song in sexual isolation between different populations or species by exposing females to wingless males and then playing different types of songs. The large role of love songs has been confirmed in these studies where male mating success was restored or increased by playback of songs of the same population or species. For example, this is the case for two other members of the *D. repleta* group, *D. mojavensis* and *D. arizonae* (Byrne 1999).

PHYLOGENY OF *D. BUZZATII* CLUSTER SPECIES

The species of the *D. buzzatii* cluster belong to the large *D. repleta* group (Ruiz and Wasserman 1993; Durando et al. 2000; Oliveira et al. 2012) including *D. buzzatii*, *D. koepferae, D. serido*, *D. borborema*, *D. seriema*, *D. antonietae*, and *D. gouveai*. All are close- ly related "sibling" species that form a monophyletic group (Manfrin and Sene 2006). These species are endemic to South America, except for *D. buzzatii* that has a cosmopolitan distribution due to its association with species of *Opuntia* cactus that have been propagated around the world for fruit production (Wasserman 1992).

The monophyly of the *D. buzzatii* cluster was first defined on the basis of a complex arrangement of chromosomal inversions (Ruiz and Wasserman 1993), yet only four fixed inversions can be used for species identification (Ruiz et al. 2000; Manfrin et al. 2001) (Figure 21). Other traits including mtDNA COI gene sequences (Manfrin, et al. 2001) and Xanthine dehydrogenase (*Xdh*) nucleotide sequences (Rodriguez-Trelles et al. 2000), as well as wing morphology (Moraes et al. 2004) have also been used to infer phylogenetic relationships among these species. Phylogenetic analysis using mtDNA COI indicated that the *D. buzzatii* cluster was a well-supported monophyletic group (Manfrin, et al. 2001; de Brito et al. 2002), but these mtDNA sequences did not help to resolve the pattern of species relationships within this group. At present, sequence variation in the *period* (*per*) gene (Franco et al. 2010) has produced a phylogeny that best resolves these branching patterns. The *per* phylogeny also reinforced the monophyletic nature of this cluster, but more importantly it resolved the relationships of *D. gouveai*, *D. borborema* and *D. seriema*, which had been difficult to understand when chromosomal inversions and mtDNA sequences were used.

HOST CACTUS, BIOGEOGRAPHY AND ECOLOGY OF *D. BUZZATII* CLUSTER SPECIES

The *D. buzzatii* cluster species are distributed over a vast geographical area in South America, ranging from northeastern Brazil to Paraguay, Bolivia and Argentina (Figure 22).

The vegetation in these areas includes the morphoclimatic biomes of caatinga (thorny scrub), cerrado (savannah), Atlantic forest, and Chaco. Like other cactophilic species belonging to the *mulleri* subgroup of the *D. repleta* group, *D. buzzatii* cluster species use fermenting cactus tissues as feeding and breeding substrates, and the level of host specificity varies among the different species (Sene et al. 1982; Ruiz, et al. 2000; Kokudai et al. 2011).

The ecology and biogeography of the *D. buzzatii* cluster species, as well as varying levels of genetic divergence within this clade make the *D. buzzatii* cluster an ideal system to address questions regarding how mate recognition systems evolve in the early stages of species divergence. First, we recorded and described the courtship songs of these species and used a comparative approach to assess whether courtship song evolution was correlated with species divergence. Our results revealed strong species-specific differentiation in multiple acoustic characteristics of male courtship songs signifying rapid evolution in this central component of acoustic courtship signaling.

DESCRIPTION OF COURTSHIP SONGS AND COMPARATIVE METHODS

We recorded the courtship songs of all seven species of the *D. buzzatii* cluster and quantified variation in courtship song components (Figure 22). Fly stocks and handling procedures are described in Oliveira et al. (2011). All flies were aged at least 8 days before use to ensure sexual maturity (Bizzo 1983; Moraes 1992). Courtship songs of ten males of each species were recorded with an ultra-sensitive microphone (Bennet-Clark 1984) in an acrylic chamber (3 x 3 x 1 cm^3) as described by Sene and Manfrin (1998). Each male was housed with two virgin females of the same species and the wings were removed from the females prior to recording. Temperature inside the recording chamber was monitored continuously with a digital thermometer because courtship songs can be temperature dependent (Byrne 1999; Ritchie et al. 2001). The time of day of recording was not controlled for. Approximately three minutes of song were recorded for each male. We digitized the song recordings at 8 KHz using Sonic Sound Forge software (2006, Creative Software Inc., Madison, Wisconsin, USA).

We analyzed courtship song components with Raven Pro 1.3 software (2003, Cornell Laboratory of Ornithology, Ithaca, New York, USA). All song measurements were made directly from the waveform tracings from Raven. For each male, five bursts of each type of song (i.e. primary and secondary) were analyzed but not all males produced a secondary song. A total of six song components was analyzed for each type of song including burst duration (BD), carrier frequency (CF), pulse number (PN), and inter-burst interval (IBI) that were measured from five randomly selected bursts. For pulse length (PL) and inter-pulse interval (IPI) five randomly selected pulses or inter-pulses, respectively, were measured per burst (Table 9, Figure 20).

Song differences among species were assessed for all song variables using PROC GLM (SAS Institute Inc. 2004) and temperature effects during recording were evaluated with analysis of covariance (ANCOVA). Species were considered a fixed effect and temperature was log transformed to improve normality. Principal components analysis (PCA) was used to reduce the dimensionality of the data in PROC PRINCOMP, and canonical discriminant function analysis (CDF) was used to help visualize species differences with PROC

CANDISC. We used both data corrected for temperature variation and the residuals in PCA and CDF analysis. Principal Components (PCs) and canonical variates (CVs) were later used for character mapping analysis (see below).

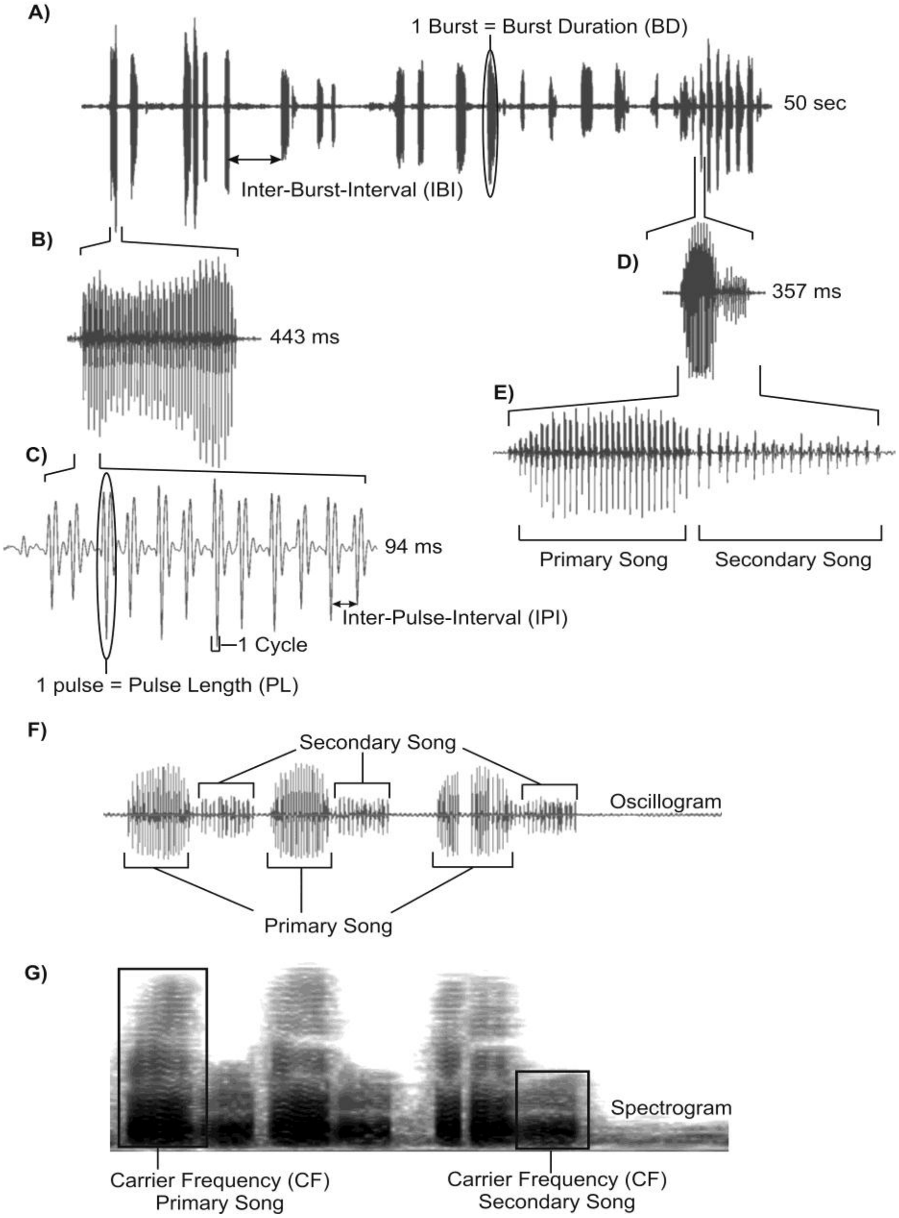


Figure 20 A-G. Typical courtship song of *D. buzzatii* species composed of pulses arranged into bursts. Oscillograms are used to illustrate the *Drosophila* courtship song terminology. (A) Fifty seconds of pulse song showing both primary and secondary song. IBI = inter-burst interval. (B) Single burst of primary song composed of 52 pulses. (C) Expanded view of B showing the first 12 polycyclic pulses. IPI = inter-pulse interval. (D) Two bursts showing primary and secondary song, respectively. (E) Enlarged view of D. (F) Oscillogram of six bursts: three bursts of primary song intercalated by three bursts of secondary song. (G) Spectrogram of the oscillogram showed in F. Bursts of primary song present higher frequency than the bursts of secondary song.

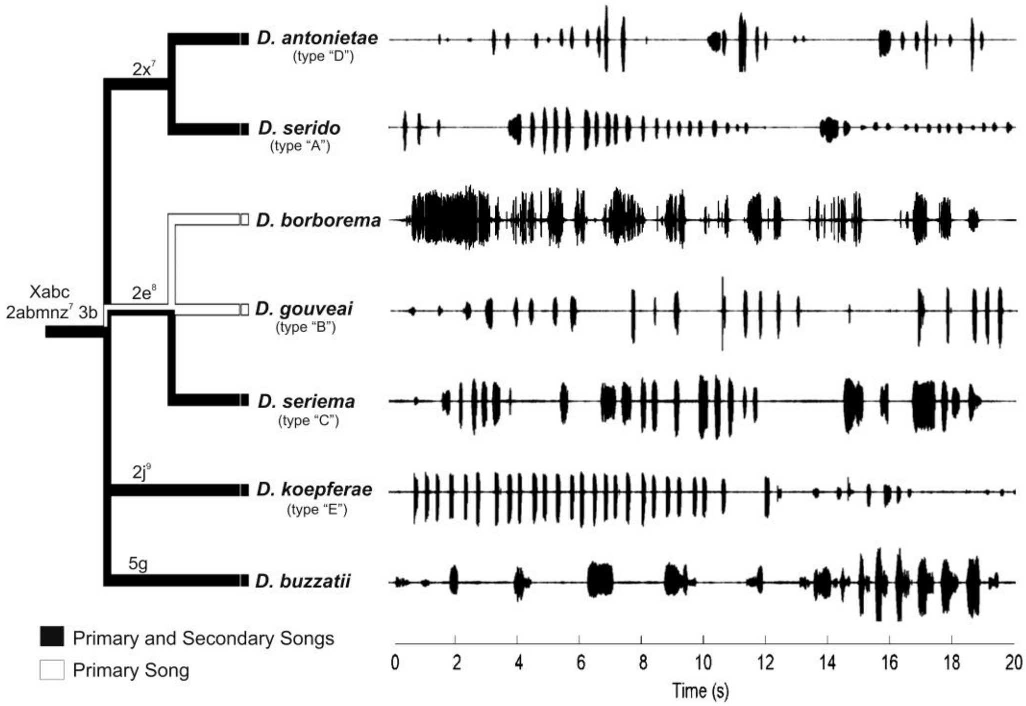


Figure 21. Left side: Consensus phylogeny based on chromosomal inversions for *D. buzzatii* species cluster. Male genitalia (type A – E) are according to Silva and Sene (1991). Chromosomal inversions, shown above the tree branches, were based on the work of Ruiz et al. (1997; 2000). Black branches characterize species that have both primary and secondary song, while white branches represent species that possess only primary song, i.e. have lost secondary song. Right side: Typical wave pattern of the male courtship songs of the species of the *D. buzzatii* cluster.

Phylogenetic Reconstruction

Phylogenetic relationships for the seven *D. buzzatii* cluster species were reconstructed using chromosomal inversion differences (Ruiz et al. 1997; Ruiz, et al. 2000) and nucleotide variation in a 443 bp fragment of the X linked *period* (*per*) gene (Franco, et al. 2010). Two outgroup species, *D. mojavensis* and *D. hydei* were also included. Because no song data were available for *D. hydei* this species was removed before the tree was used for phylogenetic analysis of song evolution. Three song components were available for *D. mojavensis*, i.e. BD, IBI, and IPI, from Etges et al. (2007). Because we were also interested in the song evolution for *D. mojavensis* as well as its effects as an outgroup, we kept this species in the character reconstruction analysis when individual song components were mapped onto the phylogeny, but removed it when PCs or CVs were used (see below).

Phylogenetic analysis using the combined data was performed using PAUP* 4.0 (Swofford 2000) as in Oliveira et al. (2011). Maximum parsimony was used to search for optimal tree(s) and heuristic searches were carried out with 100 random addition analyses and tree bisection reconnection (TBR) branch swapping. Nodal support was obtained using bootstrap analysis (1,000 replicates).

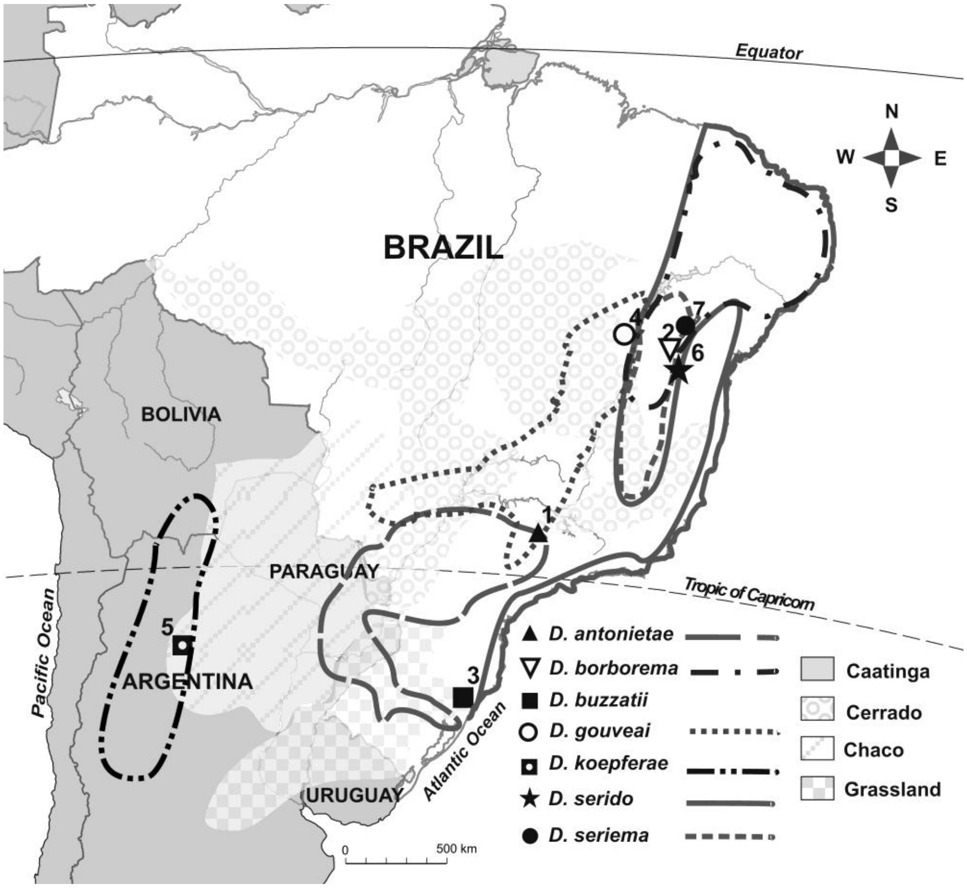


Figure 22. Partial view of South American map showing the geographic distribution of the species of the D. *buzzatii* cluster and the four major vegetation types with which these species are associated. The distribution of D. *buzzatii* is not marked because this species is found in all areas where the other species occur. Numbers represent the description for each of the species used in the courtship song analysis, i.e. species name, stock number, locality and year of collection. (1) D. antonietae (J41P1M), Serrana, São Paulo, 1999; (2) D. borborema (N70), Junco do Seridó, Paraíba, 2008; (3) D. *buzzatii* (J26A45), Osório, Rio Grande do Sul, 1998; (4) D. gouveai (J78M1), Ibotirama, Bahia, 2001; (5) D. koepferae (B20D2), Tapia, Tucumán; (6) D. serido (J92A91M), Milagres, Bahia, 2002; (7) D. seriema (D73C5BM), Morro do Chapéu, Bahia, 1990. Except for D. koepferae, from Argentina, all other species were collected in Brazil.

Mapping Song Traits onto the Phylogeny

Patterns of courtship song evolution were inferred by mapping individual song traits, i.e. BD, IBI, and IPI, Principal Components (PCs), and canonical variates (CVs) onto the reconstructed phylogeny using Mesquite 2.74 (Maddison and Maddison 2010). Because some song components were temperature dependent (e.g. Ritchie, et al. 2001; Etges, et al. 2007), all six song components were regressed against temperature using PROC REG to generate predicted (PRD) and residual (RES) values used in character mapping. These values were mapped onto the first of two most parsimonious trees instead of the strict consensus tree because one of the models used, Squared Change Parsimony Gradual (see below), requires branch length information. Character reconstruction analysis was used to infer phylogenetic signal and was performed using three parsimony methods, Linear Parsimony (LP), Squared Change Parsimony Gradual (SCPG), and Squared Change Parsimony Punctuated (SCPP). In addition to these three parsimony methods, we also used the test for serial independence (TFSI) to detect phylogenetic signal as described in Abouheif (1999) using Phylogenetic Independence 2.0 (Reeve and Abouheif 2003). Phylogenetic signal was used as a measure of congruence between the phylogeny and variation in the song variables.

Table 9. Definition of song parameters analyzed in the species of the D. buzzatii cluster

Song Parameter	**Abbreviation**	**Unit**	**Definition**
Burst Duration	BD	Milliseconds (ms)	The time between the first and last pulse in a burst.
Carrier frequency	CF	Hertz (Hz)	Highest peak frequency from a fast Fourier transformation.
Pulse Length	PL	Milliseconds (ms)	The length of a pulse.
Pulse Number	PN	----	Number of pulses per burst.
Inter-Burst Interval	IBI	Milliseconds (ms)	The time between the end of a burst and the beginning to the next one.
Inter-Pulse Interval	IPI	Milliseconds (ms)	The time between pulses, measured from peak-to-peak.

For each male five bursts were analyzed for each type of song (primary and secondary song).

We tested the null hypothesis that courtship songs have evolved independently of species evolution due to non-phylogenetic influences such as developmental noise, ecological effects (e.g. rearing conditions), or species-specific sexual selection. Our alternative hypothesis was that positive phylogenetic signal should be observed due to the phylogenetic affinities of these species and song traits. The presence of phylogenetic signal was tested with all three parsimony methods by randomly modifying the most parsimonious tree, named here as a reference tree (Oliveira, et al. 2011). The terminal taxa on the reference tree were reshuffled 10,000 times to generate a population of random trees for each of the variables tested, i.e. PCs, CVs, and individual variables (BD, IBI, and IPI). These random trees with reshuffled taxa were then compared with the reference tree to test whether the mapped variables were more conserved than expected by chance alone. Presence of phylogenetic signal was inferred if the number of parsimony character steps in the reference tree was less than in 95% of the trees with reshuffled taxa and fell on the extreme left of the distribution. For all three parsimony methods and TFSI, *P* values were corrected for multiple comparisons via false discovery rate (FDR) analysis (Benjamini and Hochberg 1995; Laurin et al. 2009.

Differences in Courtship Song Components

Male courtship songs consisted of low-frequency, polycyclic pulses arranged into pulse trains or bursts (Figures 20 and 21). Courtship songs were produced by vibration of both wings during courtship and until copulation, but no song was produced during or after copulation. Primary song was produced during most of the courtship sequence and secondary songs were usually produced later in courtship, immediately before copulation. Secondary song was absent in males of *D. borborema* and *D. gouveai*. Ambient recording temperature ($\overline{X}$ ± SD = 25.33 ± 1.01°C, *N* = 70, range 23 – 27°C) had little significant effect on variation in any of the song components except for primary song IPI (Table 10). ANCOVA revealed heterogeneity of slopes for a few song components caused by different species. Along with some missing data and only 10 males per species recorded, we observed differences in significance between Type I and Type III sums of squares for species differences (results not shown) and statistical significance for the overall model sums of squares. We report Type III sums of squares and their significance in Table 10 to be conservative, but Type I sums of squares for BD, CF, PN, IBI, and IPI were all statistically significant. Further, significant pair-wise species differences were observed when least square means were analyzed (See Figure 23 and below). As the ANCOVAs used contained one fixed effect (species), temperature as a covariate, and a species X temperature interaction term, we concluded that Type I sums of squares were appropriate for revealing species differences in these song components. Differences among species as well as differences in type of song for each song component are described below. Since *D. borborema* and *D. gouveai* lacked secondary songs, no comparison for type of song was available for these species.

Pair-wise comparisons using least square means revealed that burst duration was variable for several of the species pairs (Figure 23A). Furthermore, BD did not vary consistently for individuals of the same species or even in the same individual. In fact, song bursts in the same individual had different shapes, amplitudes and durations (Figure 21). Mean BD for primary song ranged from 129.34 ms to 660.44 ms, and for secondary song, ranged from 78.67 ms to 440.17 ms (Table 11). Except for *D. borborema*, CF was relatively similar among the other species (Figure 23B). For all species, CF was characterized by low frequency peaks with mean CF for primary song ranging from 213.13 Hz to 467.51 Hz, and for secondary song ranging from 274.67 Hz to 379.69 Hz (Table 11). Pulse length or pulse duration was more conserved for primary song than for secondary song (Figure 23C). All seven species produced songs with polycyclic pulses consisting of two to four cycles per pulse. Mean PL for primary song ranged from 5.25 ms to 6.14 ms, and for secondary song, from 6.22 ms to 7.75 ms (Table 11).

Pulse number influenced burst duration. For instance, *D. borborema* produced long bursts (Figure 23A), which had more pulses (Figure 23D). Mean PN for primary song ranged from 9.6 pulses to 42.3 pulses (Table 11). For secondary song, mean PN ranged from 5.8 pulses to 24.3 pulses (Table 12). Because of its correlation with burst duration, pulse number was also highly variable. Inter-burst interval, measured as the distance between bursts, was difficult to calculate because some males stopped and started singing multiple times. Least square mean comparisons revealed that *D. borborema* had the highest IBI values. Even though IBI for secondary song was not statistically different among species (*P* = 0.2482), there was variation among individuals of the same species, especially for *D. antonietae*, as indicated by a large standard error (Figure 23E). Mean IBI for primary song ranged from 347.56 ms to 1348.22 ms, and for secondary song, from 480.73 ms to 1536.84 ms (Table 11).

Based on least square mean comparisons for primary song, *D. borborema* had the highest mean IPI, which was significantly different from all other species (Figure 23F). For secondary song, *D. buzzatii*, *D. koepferae*, and, *D. seriema* had the highest mean IPI followed by *D. serido* and lastly by *D. antonietae*. Mean IPI for primary song ranged from 7.92 ms to 14.20 ms, and for secondary song, from 8.85 ms to 12.30 ms (Table 11). Differences in IPI between primary and secondary songs were significant among the five species that possessed both types of songs ($P = 0.0113$). Significant pair-wise species differences were observed when least square means were analyzed, but only *D. buzzatii* showed significant differences for both song types where IPIs were shorter for primary song and longer for secondary song (Figure 24). Therefore, except for *D. buzzatii,* the other four species had unimodal IPIs, i.e. just one type of IPI. Bimodal IPIs, i.e. short and long IPIs, are considered a characteristic of the *D. repleta* group (Ewing and Miyan 1986).

Principal components analysis (PCA) revealed that the five principal components (PCs) accounted for 96% of the variation in the data for the seven species. The first principal component (PC1) accounted for 51% of the variance and was mainly driven by the differences between primary and secondary songs. PC1 scores were all negative for primary song traits (except for carrier frequency), and positive for secondary song traits (Table 12). Such differences were accentuated because secondary song was absent in some males and completely absent in *D. borborema* and *D. gouveai*. Accordingly, PC1 separated these two species from the others (Figure 25). The second principal component (PC2) accounted for 20% of the variation and separated species largely based on differences in primary songs traits, i.e. BD, CF, PN, and IBI. The third and fourth PCs, which represented 16% and 6% of the variation, respectively, were also mostly influenced by differences in primary songs (Table 12).

Canonical discriminant function (CDF) analysis using the residuals of the song characters yielded significant multivariate differences among species (Wilks $\lambda = 0.0000$, $F = 6.13 \times 10^{11}$, $P < 0.0001$). The first three canonical variables accounted for 98% of the total variation in courtship songs. As observed with PC analysis, the first canonical variate (CV1) was largely influenced by differences in type of song, primary and secondary, and the second and third canonical variates (CV2 and CV3) expressed differences among species as a result of variation in primary song (results not shown). Altogether, the results from PCA and CDF analysis confirmed that courtship songs were species-specific in the *D. buzzatii* cluster and primary song was mainly responsible for species differences.

CHARACTER MAPPING ANALYSIS OF COURTSHIP SONG

We used the first of two most parsimonious trees to perform the character reconstruction analysis (Figure 26). No phylogenetic signal was observed for any of the song traits mapped onto the phylogeny using either temperature corrected data or the residuals, i.e. individual song traits (BD, IBI, and IPI), CVs or PCs, using four different reconstruction methods (Table 13). Even though *D. mojavensis* is not closely related to the *D. buzzatii* cluster, this species had long bursts similar to *D. buzzatii*, *D. serido*, and *D. seriema*. However, *D. koepferae*, closely related to *D. buzzatii,* had short bursts (Figure 26A). Furthermore, *D. borborema* and *D. seriema* are closely related, but the former had the longest bursts of all species (Table 11, Figure 26). Similar differences were observed for IBI and IPI (Figure 26B, C) and the other variables (PCs and CVs). When PCs and CVs were mapped onto a phylogeny with or without *D. mojavensis* as an outgroup, phylogenetic signal was not detected (Table 13) indicating that this species did not influence the results. Overall, our results demonstrated no congruence between species differences in these song traits and phylogenetic structure in this clade of *Drosophila* species.

Table 10. Results of ANCOVAs for six song parameters analyzed for the species of the *D. buzzatii* cluster

Song Parameter	**Source of Variation**	***df***	**Type III SS**	***F***	***P***
Primary Song					
Burst Duration (BD)	Model	12	2227914.86	9.73	<0.0001
	Species	5	34867.21	0.37	0.8701
	Lgtemp	1	8456.86	0.44	0.5083
	Lgtemp x Species	5	36531.98	0.38	0.8585
	Error	57	1087763.35		
Carrier Frequency (CF)	Model	12	470873.61	10.70	<0.0001
	Species	5	10812.81	0.59	0.7077
	Lgtemp	1	5316.89	1.45	0.2335
	Lgtemp x Species	5	10980.11	0.60	0.7008
	Error	57	208968.10		
Pulse Number (PN)	Model	12	10326.37	10.11	<0.0001
	Species	5	292.94	0.69	0.6341
	Lgtemp	1	187.01	2.20	0.1437
	Lgtemp x Species	5	306.34	0.72	0.6110
	Error	57	4849.79		
Pulse Length (PL)	Model	12	8.92	1.28	0.2576
	Species	5	2.37	0.81	0.5452
	Lgtemp	1	0.05	0.08	0.7801
	Lgtemp x Species	5	2.36	0.81	0.5466
	Error	57	33.19		
Inter-Burst-Interval (IBI)	Model	12	12948801.41	2.43	0.0126
	Species	5	1582033.81	0.71	0.6164
	Lgtemp	1	43479.23	0.10	0.7555
	Lgtemp x Species	5	1585975.11	0.71	0.6151
	Error	57	25303658.70		
Inter-Pulse-Interval (IPI)	Model	12	295.59	41.76	<0.0001
	Species	5	4.00	1.36	0.2549
	Lgtemp	1	5.27	8.93	0.0041
	Lgtemp x Species	5	3.57	1.21	0.3157
	Error	57	33.62		
Secondary Song					
Burst Duration (BD)	Model	9	542983.86	1.72	0.1285
	Species	4	1083.37	0.01	0.9999
	Lgtemp	1	72.66	0.00	0.9640
	Lgtemp x Species	4	798.02	0.01	0.9999
	Error	30	1053962.14		

Table 10. (Continued)

Song Parameter	**Source of Variation**	***df***	**Type III SS**	***F***	***P***
Secondary Song					
Carrier Frequency (CF)	Model	9	70692.77	1.43	0.2216
	Species	4	7335.99	0.33	0.8537
	Lgtemp	1	6278.32	1.14	0.2943
	Lgtemp x Species	4	7367.70	0.33	0.8527
	Error	30	165310.43		
Pulse Number (PN)	Model	9	1406.84	1.46	0.2095
	Species	4	18.75	0.04	0.9962
	Lgtemp	1	3.49	0.03	0.8581
	Lgtemp x Species	4	17.90	0.04	0.9965
	Error	30	3220.48		
Pulse Length (PL)	Model	9	28.33	0.73	0.6794
	Species	4	16.08	0.93	0.4593
	Lgtemp	1	4.74	1.10	0.3030
	Lgtemp x Species	4	15.95	0.92	0.4635
	Error	30	129.58		
Inter-Burst-Interval (IBI)	Model	9	24012175.07	1.70	0.1335
	Species	4	8993207.14	1.43	0.2482
	Lgtemp	1	151204.19	0.10	0.7586
	Lgtemp x Species	4	9094390.70	1.45	0.2432
	Error	30	47160005.47		
Inter-Pulse-Interval (IPI)	Model	9	66.66	2.08	0.0637
	Species	4	9.01	0.63	0.6425
	Lgtemp	1	2.25	0.63	0.4323
	Lgtemp x Species	4	8.84	0.62	0.6505
	Error	30	106.61		

See Figure 23 for least square mean differences between species. Lgtemp = $\log_{10}$ temperature.

EVOLUTION OF COURTSHIP SONGS

No phylogenetic signal was observed when we mapped quantitative song traits onto an independently derived phylogeny of the *D. buzzatii* cluster (Table 13, Figure 26) consistent with rapid evolution of male courtship songs. Although rates of species divergence in this group given their genetic affinities have been well described (e.g. Manfrin and Sene 2006), our analyses need to be broadened to a larger sampling of *D. repleta* group species in order to assess the validity and generality of our conclusions. Few comparative studies have investigated the evolution of behavioral traits involved in mate recognition and reproductive isolation (e.g. Ewing and Miyan 1986; Kusmierski et al. 1997; Gleason and Ritchie 1998; Henry et al. 1999; Etges and Noor 2002; Symonds and Elgar 2004; Grace and Shaw 2012). Even scarcer are studies that have mapped quantitative variation, rather than categorical differences, in behavioral traits onto a phylogeny. This is in part because it has been difficult to identify sufficient numbers of species clusters whose members are in different stages of reproductive isolation and for which there is comparative data for phenotypes involved with pre- and/or postmating isolation. Furthermore, the long-standing view that the evolution of behavioral traits is weakly or uncorrelated with phylogeny (e.g. Atz 1970; Baroni Urbani 1989; Blomberg et al. 2003) has certainly contributed to *a priori* view that all behavioral traits are labile.

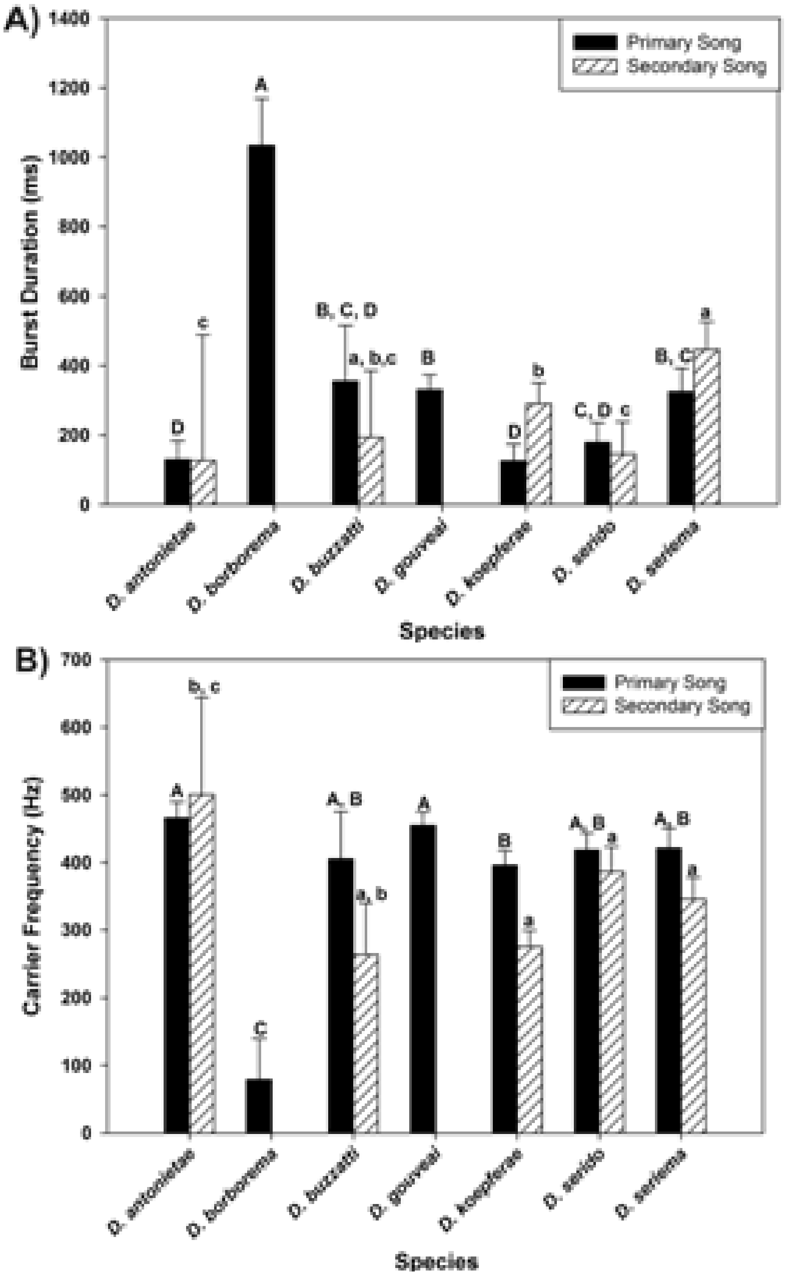
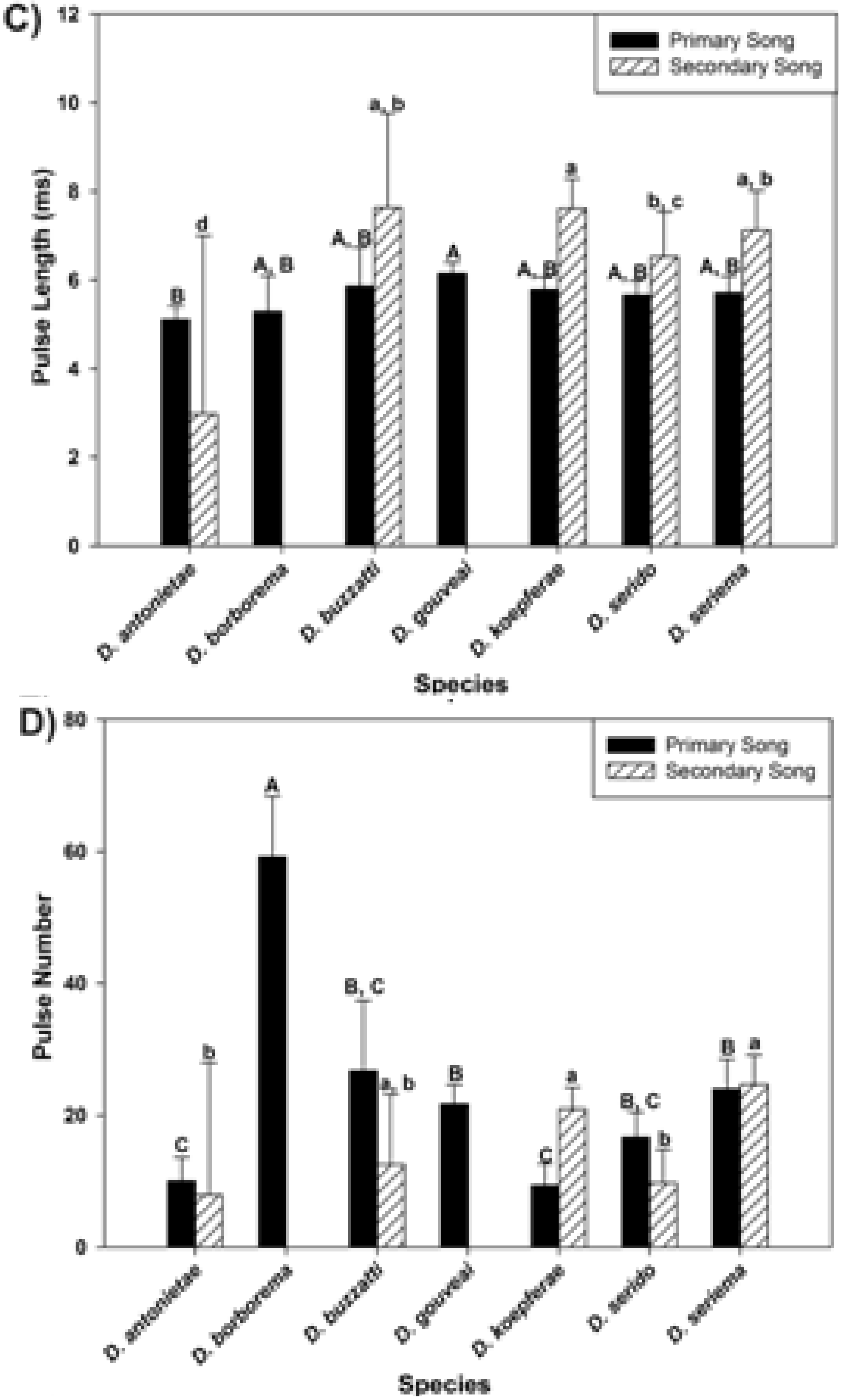


Figure 23. (Continued)


C)
Primary Song
Secondary Song
Pulse Length (ms)
0
2
4
6
8
10
12
B
d
A, B
A, B
a, b
A
A, B
a
A, B
b, c
A, B
a, b
D. antonietae
D. borborema
D. buzzatii
D. gouveai
D. koepferae
D. serido
D. seriema
Species
D)
Primary Song
Secondary Song
Pulse Number
0
20
40
60
80
C
b
A
B, C
a, b
B
C
a
B, C
b
B
a
D. antonietae
D. borborema
D. buzzatii
D. gouveai
D. koepferae
D. serido
D. seriema
Species

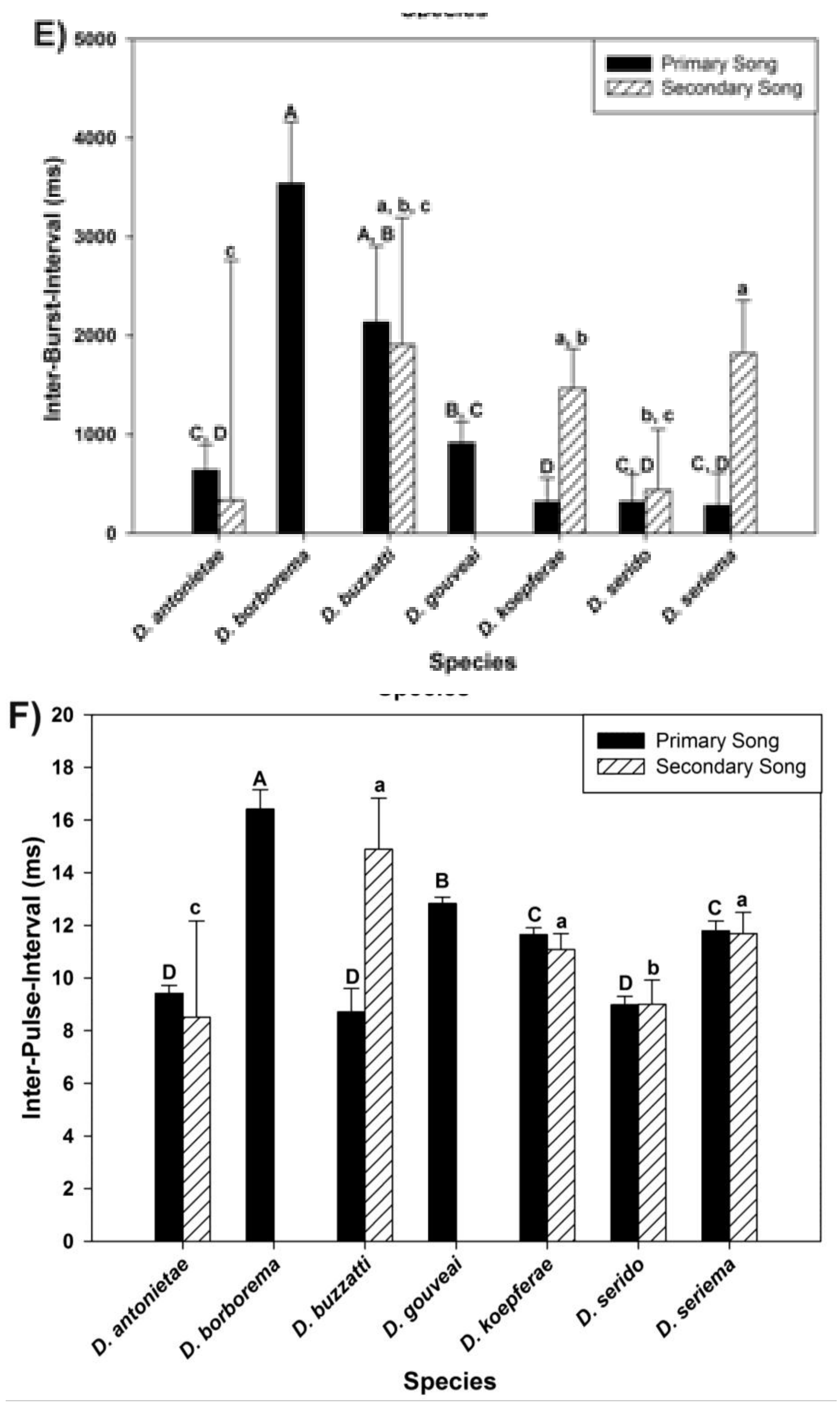


Figure 23 A-F. Least square means ± SE of six song components. A) Burst duration, B) Carrier frequency, C) Pulse number, D) Pulse length, E) Inter-burst-interval, and F) Inter-pulse-interval. Nonsignificant means share the same letter. Interspecific comparisons were performed for primary and secondary song separately.

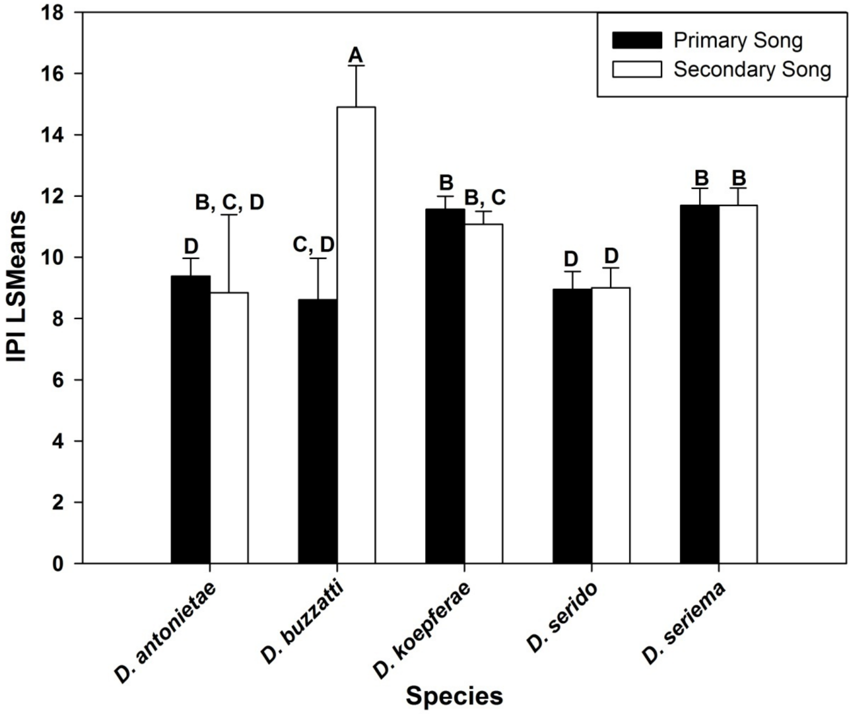


Figure 24. Differences in IPI, inter-pulse interval, between primary and secondary songs among five species using least square means ± SE. No comparison was performed for *D. borborema* and *D. gouveai* because these species lacked secondary song. Nonsignificant means share the same letter.

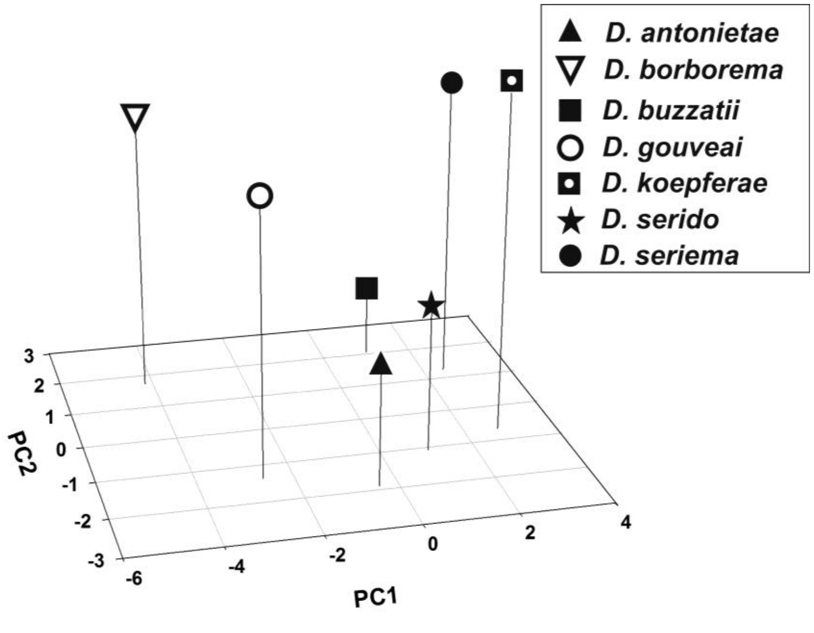


Figure 25. Three dimensional plot of the D. *buzzatii* species cluster based on the first three principal components (PCs) obtained from six song components (see Table 9). Altogether, the first three PCs explained 87% of the variance in the data (PC1 = 51%, PC2 = 20%, and PC3 = 16%).

Table 11. Mean ± SD of courtship song parameters of the D. buzzatii species cluster

		Burst Duration (ms)			**Carrier Frequency (Hz)**			**Pulse Length (ms)**			**Pulse Number**			**Inter-burst-interval (ms)**			**Inter-pulse-interval (ms)**		
Species		**Mean**	**SD**	***n***	**Mean**	**SD**	***n***	**Mean**	**SD**	***n***	**Mean**	**SD**	***n***	**Mean**	**SD**	***n***	**Mean**	**SD**	***n***
D. antonietae	PS	142.72	45.45	50	447.51	51.14	50	5.25	0.60	250	10.82	3.91	50	588.26	272.69	50	9.52	0.40	250
	SS	78.67	7.36	50	359.34	60.43	50	6.48	1.50	250	5.77	1.30	50	501.35	149.60	50	9.90	0.90	250
D. borborema	PS	660.44	249.12	50	213.13	111.32	50	5.54	1.20	250	38.58	13.63	50	1312.00	1295.20	50	14.20	1.30	250
	SS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D. buzzatii	PS	438.74	121.28	50	467.51	34.71	50	5.29	0.70	250	42.30	11.13	50	1348.80	666.46	50	7.92	1.00	250
	SS	201.22	20.47	50	324.30	105.78	50	7.75	0.70	250	13.67	1.76	50	791.85	695.21	50	12.30	1.50	250
D. gouveai	PS	330.2	117.28	50	456.26	52.28	50	6.14	0.60	250	21.80	7.62	50	938.48	765.76	50	12.80	1.10	250
	SS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D. koepferae	PS	129.34	33.91	50	396.87	43.24	50	5.92	0.90	250	9.62	2.73	50	350.60	107.25	50	11.50	0.70	250
	SS	290.18	68.58	50	274.67	30.09	50	7.42	3.20	250	20.63	8.68	50	1536.80	2351.90	50	11.10	2.20	250
D. serido	PS	189.26	85.49	50	425.00	27.01	50	5.41	0.50	250	17.08	7.46	50	347.56	130.95	50	9.19	0.50	250
	SS	145.48	94.84	50	379.69	45.15	50	6.22	1.70	250	11.28	6.06	50	480.73	180.28	50	8.95	1.10	250
D. seriema	PS	385.42	154.76	50	417.51	56.12	50	5.64	0.60	250	28.10	11.48	50	534.84	466.07	50	11.40	0.50	250
	SS	440.17	343.09	50	347.04	83.39	50	7.27	1.70	250	24.28	16.91	50	1250.70	666.56	50	11.6	2.30	250

ms = milliseconds; Hz = Hertz; *N* = number of flies recorded, i.e. 10 males per species; *n* = number of sample size of each parameter; SD = standard deviation; PS = primary song; SS = secondary song.

Table 12. Eigenvectors from a principal component analysis for six courtship song traits

Variable	**PC1 (51%)**	**PC2 (20%)**	**PC3 (16%)**	**PC4 (6%)**	**PC5 (3%)**
BD – PS	**-0.233**	**0.516**	0.063	-0.003	0.091
CF – PS	**0.203**	**-0.268**	**-0.329**	**0.620**	**0.394**
PN – PS	-0.135	**0.569**	-0.154	0.180	0.061
PL – PS	-0.017	-0.127	**0.565**	**0.592**	**-0.429**
IBI – PS	**-0.240**	**0.394**	-0.184	**0.386**	-0.130
IPI – PS	**-0.221**	0.040	**0.571**	-0.159	0.146
BD – SS	**0.351**	**0.202**	0.204	-0.011	**0.424**
CF – SS	**0.375**	0.112	-0.120	-0.138	**-0.331**
PN – SS	**0.367**	0.170	0.170	-0.070	**0.250**
PL – SS	**0.377**	0.159	-0.056	-0.056	**-0.353**
IBI – SS	**0.309**	0.151	**0.310**	0.176	**0.225**
IPI – SS	**0.379**	0.187	-0.033	0.035	**-0.289**

Variables were generated using values corrected for temperature variation. Only the first five principal components (PC1 – PC5) are shown that accounted for 96% of the variation in the data. Values in parentheses are the percentages of the variance explained by each PC. Numbers in bold represent the song traits that most contributed to the PC scores. See Table 9 for description of song traits. BD = burst duration; CF = carrier frequency; PN = pulse number; PL = pulse length; IBI = inter-burst interval; IPI = inter-pulse interval; PS = primary song; SS = secondary song.

Table 13. Analysis of congruence between the chromosomal inversion plus *per* gene phylogeny and courtship song data

	Parsimony methods									**Test for Serial Independency (TFSI)**	
	Linear parsimony (LP)			**Squared change parsimony gradual (SCPG)**			**Squared change parsimony punctuated (SCPP)**				
Characters	*Reference Tree*	*Random Trees*	*P*	*Reference Tree*	*Random Trees*	*P*	*Reference Tree*	*Random Trees*	*P*	Observed Mean C-Statistics	*P*
BD-PRD	926.13	879.83	0.5409	30856.68	31145.87	0.5709	140094.71	133425.24	0.6271	-0.0538	0.3940
BD-RES	181.61	177.43	0.5901	2668.23	1954.91	0.7543	9250.87	8415.69	0.5844	-0.1063	0.4740
IBI-PRD	4.64	4.29	0.5467	0.79	0.56	0.8254	2.92	2.43	0.7834	-0.1547	0.2670
IBI-RES	0.23	0.23	0.4710	0.01	0.006	0.7441	0.03	0.03	0.8253	-0.1316	0.1160
IPI-PRD	26.26	25.30	0.4487	16.86	19.92	0.4412	79.62	85.67	0.3648	0.0663	0.2870
IPI-RES	1.31	1.27	0.7381	0.17	0.09	0.9409	0.47	0.40	0.7327	-0.1535	0.2330
CV1-PRD	314.69	389.05	0.1079	3372.06	6074.17	0.0794	15141.00	24097.36	0.0896	0.2304	0.2010
CV1-RES	2.37	3.62	0.0438	0.31	0.57	0.1065	1.62	2.25	0.1107	0.18	0.2760
CV2-PRD	164.95	177.71	0.2599	2366.06	1406.55	0.8578	5365.56	5527.05	0.4406	0.0411	0.4800
CV2-RES	1.94	1.78	0.5990	0.13	0.14	0.5272	0.78	0.54	0.9838	-0.33	0.0530
CV3-PRD	53.59	53.61	0.1685	163.13	190.85	0.5152	654.49	761.93	0.1769	0.0466	0.2650
CV3-RES	1.22	1.50	0.1358	0.08	0.09	0.5113	0.26	0.34	0.1771	0.18	0.1340
PC1-PRD	11.99	12.40	0.2345	12.25	6.76	0.9075	27.98	26.91	0.5137	-0.0506	0.4750
PC1-RES	10.00	10.94	0.1032	3.01	5.38	0.1255	22.00	21.33	0.5395	-0.05	0.5430
PC2-PRD	7.07	8.18	0.2973	1.51	2.68	0.1380	9.32	10.57	0.3282	0.0654	0.4460
PC2-RES	4.00	4.00	0.5000	0.53	1.55	0.0464	8.42	6.13	0.9061	-0.24	0.1020
PC3-PRD	6.02	6.88	0.2060	0.90	1.99	0.0472	8.68	7.87	0.5831	-0.0230	0.4070
PC3-RES	8.00	7.42	0.4673	3.53	2.55	0.8553	10.64	10.06	0.6180	-0.12	0.1860
PC4-PRD	4.35	4.02	0.9084	0.74	0.73	0.6158	3.20	2.89	0.6300	-0.1597	0.2760
PC4-RES	15.00	13.94	0.4607	4.74	9.43	0.0514	44.00	37.26	0.8321	-0.14	0.2390
PC5-PRD	1.48	1.66	0.1448	0.24	0.15	0.8154	0.54	0.57	0.4503	0.0180	0.4250
PC5-RES	11.00	11.83	0.1625	3.83	5.73	0.9551	19.76	22.64	0.2994	0.05	0.5580

Table 13. (Continued)

Individual song components, i.e. burst duration (BD), inter-burst interval (IBI), and inter-pulse interval (IPI), were mapped onto a phylogeny that included the seven species of the D. *buzzatii* cluster plus D. movajensis as an outgroup. Furthermore, CDF analysis and PC analysis were used to obtain canonical variates (CVs) and principal components (PCs). These variables were also mapped onto a phylogeny, but this time it did not include an outgroup. All variables mapped onto the phylogeny were based on predicted (PRD) and residual (RES) values. Three different parsimony methods were used in Mesquite: linear parsimony (LP), squared-change parsimony assuming a gradual model of evolution (SCPG), and squared-change parsimony with a punctuated model of evolution (SCPP). In all three models, presence of phylogenetic signal for each character was assessed by comparing the mean parsimony character steps from the reference tree (as shown on Figure 26) with those of a population of random trees. Terminal taxa were reshuffled 10,000 times to generate the random trees. Phylogenetic signal was positive when the mean parsimony character steps for the reference tree were significantly smaller than the mean parsimony character steps for the random trees. The detection of phylogenetic signal was also examined with the test for serial independence (TFSI) run with 1,000 replicates using the program Phylogenetic Independence 2.0. P-values were adjusted using false discovery rate (FDR) analysis. No p values were significant after the adjustment.

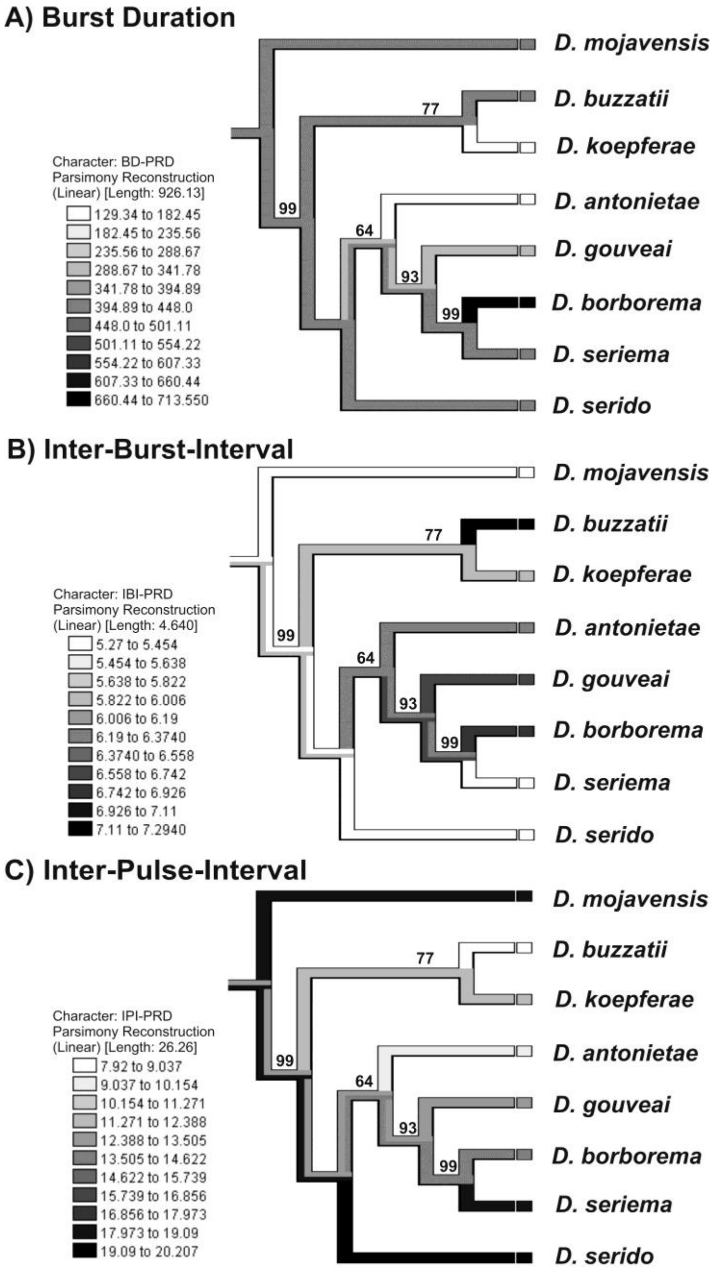


Figure 26 A-C. Phylogenetic character mapping using the linear parsimony model. This phylogeny represents a most parsimonious tree (one of two trees) of species of the *D. buzzatii* cluster inferred from chromosomal inversions (Ruiz et al. 1997; 2000) and *period* gene (Franco et al. 2011). *D. mojavensis* was used as an outgroup species. A) Burst Duration; B) Inter-Burst-Interval; and C) Inter-Pulse-Interval. Bootstrap values (shown above the nodes) were based on 1,000 replicates and 100 random additions. Only bootstrap values above 50% are shown.

In their comparative study of the courtship songs in 22 species of the *D. repleta* group, Ewing and Miyan (1986) mapped the evolution of song types, A and B songs, onto a phylogeny based on chromosomal inversions. They observed that presence of two song types was ancestral in the group, but over evolutionary time some species have lost one of the song types while in others B song has become more complex. Etges (2002) mapped song types onto the phylogeny of the group using an available species phylogeny (Durando, et al. 2000), and showed that song type evolution was not concordant with the observed phylogenetic relationships among species and has been characterized by diversification, character loss, and reversal. Contrasting results have been found in other animal species regarding the evolution of courtship songs. In the *Drosophila willistoni* group, Gleason and Ritchie (1998) observed that song divergence was variable and not correlated with genetic divergence. In green lacewings songs were homoplasic (Henry, et al. 1999; 2012), while in oropendola birds songs were more conserved (Price and Lanyon 2002).

The inclusion of more comparative data is needed to calibrate rates of song evolution. Clearly courtship songs have evolved more rapidly than species diversification in several *Drosophila* species groups, but other factors must be involved in shaping larger phylogenetic trends in song evolution. The *D. repleta* group is a potentially useful group for this analysis since it is one of the largest monophyletic groups of *Drosophila*, with over 100 species (Throckmorton 1982; Vilela 1983; Durando, et al. 2000; Oliveira, et al. 2012), and is composed of species that have been intensively studied for decades, as is the case of the species of the *D. mojavensis* and *D. buzzatii* clusters (Byrne 1999; Etges 2002; Etges, et al. 2006; Manfrin and Sene 2006; Etges, et al. 2007). Using the comparative approach in a hypothesis testing framework, assessing rates of character evolution and the influence of phylogenetic affinities for mate recognition signals should help to resolve broad-scale evolutionary trends in mating signal evolution and provide some clarity into the origins of the spectacular diversity of mate communication systems we seek to understand.

CONCLUSION

Our comparative analysis of quantitative variation in male courtship songs revealed that song evolution was uncorrelated with the phylogenetic relationships among species. Mapping primary and secondary pulse songs types onto the phylogeny revealed that the presence of two songs is ancestral in the *D. buzzatii* cluster (Figure 21). These findings are in agreement with Ewing and Miyan (1986), who suggested that two song types is a primitive character state in the *D. repleta* group. They also proposed that differences in IPI (short and long IPIs) were responsible for the differences between A and B songs in species of the *D. repleta* group. We did not find clear correspondence between A and B songs and our designations of primary and secondary songs. Only *D. buzzatii* males produced significantly different primary and secondary songs, i.e. short IPI for primary song and long IPI for secondary song (Figure 24). In the other four species that possessed both types of songs, IPIs were unimodal. Ewing and Miyan (1986) also reported that *D. buzzatii* produced A song, but lacked B song. Males of the strain of *D. buzzatii* that we analyzed clearly presented two types of songs, so it is likely that there is intraspecific variation in song types for *D. buzzatii*. Intriguingly, they also described *D. mojavensis* as having only A song, but other studies have found both types of songs (Byrne 1999; Etges, et al. 2006).

The high levels of variation in courtship song among the different species of the *D. buzzatii* cluster are in agreement with other studies involving closely related *Drosophila* species (Cowling and Burnet 1981; Ewing and Miyan 1986; Hoy et al. 1988; Hoikkala et al. 1994; Tomaru and Oguma 1994; Ritchie and Gleason 1995; but see Noor et al. 2000). These findings have suggested that aspects of courtship behavior and mate recognition can be more distinct than morphology or other traits in closely related species (Butlin and Ritchie 1994; Mendelson and Shaw 2005). In fact, it is common for cryptic species to show low levels of genetic divergence in contrast to major differences in courtship behavior phenotypes (Henry et al. 2002). These patterns of differentiation are consistent with a significant role of sexual selection promoting sexual isolation and speciation (Panhuis et al. 2001). However, Noor et al. (2000) observed a lack of divergence in courtship songs between recently diverged subspecies *Drosophila pseudoobscura pseudoobscura* and *D. p. bogotana*, implying that rates of evolution in courtship songs may vary among different species groups. Also, Costa et al. (2000) observed low levels of intraspecific courtship song variation in *D. meridionalis*, also a member of the *D. repleta* group, despite the fact that karyotypic differentiation has been reported in different populations. Future studies will have to include fine-scale intraspecific, population level studies to gauge accurate rates of courtship signal and male-female signaling system evolution.

Similar to many other *Drosophila* species, courtship songs in the *D. buzzatii* cluster were characterized by low-frequency songs (Figure 20) limiting the use of male song to close-range courtship (Ewing 1983). Hawaiian *Drosophila* species are a remarkable exception, since these species produce high-frequency songs (Hoy, et al. 1988). High levels of quantitative variation were observed within *D. buzzatii* cluster species for some of the song components, e.g. burst duration and pulse number (Figure 23A, D), implying that these two traits may not be reliable species-specific signals as they would not serve as consistent species recognition signals. Furthermore, for song traits that were more species specific, e.g. pulse length and IPI (Figure 23C, F), there was significant overlap among species suggesting that females may use more than one song component during mate and/or species recognition. Despite large variation at the individual level, courtship songs, particularly primary songs, were species-specific in the *D. buzzatii* cluster (Table 10, Figure 25). Certainly, the large differences in male courtship songs are likely to play a role in interspecific sexual isolation in the *D. buzzatii* cluster (Oliveira et al., unpubl. data), but song playback experiments with wingless males (Byrne 1999) have yet to be performed.

In the *fasciola* subgroup, a basal clade of species in the *D. repleta* group, Costa and Sene (2002) reported that IPI was species-specific with little intraspecific variation, suggesting a potential role in species recognition. In *D. montana,* females prefer songs with short pulses and high carrier frequency (Ritchie et al. 2005). In *D. ananassae* and *D. pallidosa* females recognize the frequency spectra of bursts (determined by inter-pulse-interval, intra-pulse-frequency and cycles per pulse) as a species-specific signal rather than individual song components (Yamada, et al. 2002).

ACKNOWLEDGEMENTS

We thank P.R. Epifânio for technical assistance and C. H. A. S. Motta for recording the songs of *D. borborema*. F. F. Franco and A. L. H. Esguicero assisted with field collection of cactus and flies. Funding was provided by a supplement to National Science Foundation

DEB-0211125 to WJE, a Dissertation Research Award from the J. William Fulbright College of Arts & Sciences, University of Arkansas to CCO, and grants from FAPESP, CNPq and USP to MHM and FMS.

REFERENCES

Abouheif E. 1999. A method for testing the assumption of phylogenetic independence in comparative data. *Evol Ecol Res* 1: 895-909.

Atz J. W. 1970. *The application of the idea of homology to behavior.* In: Aronson L. R., Tobach E., Lehrman D. S., Rosenblatt J. S., editors. Development and Evolution of Behavior: Essays in Memory of T. C. Schnierla. San Francisco: W. H. Freeman. p. 53-74.

Baroni Urbani C 1989. Phylogeny and behavioural evolution in ants, with a discussion of the role of behaviour in evolutionary processes. *Ethol Ecol Evol* 1: 137-168.

Benjamini Y., Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc* B 57: 289-300.

Bennet-Clark H. C. 1984. A particle velocity microphone for the song of small insects and other acoustic measurements. *J Exp Biol* 108: 459-463.

Bennet-Clark H. C., Ewing A. W. 1969. Pulse interval as a critical parameter in the courtship song of *Drosophila melanogaster*. *Anim Behav* 17: 755-759.

Bizzo N. M. V. 1983. *Estudos sobre a biologia e isolamento reprodutivo em Drosophila serido*. [M.S.]. [São Paulo]: Universidade de São Paulo.

Blomberg S. P., Garland T., Jr., Ives A. R. 2003. Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution* 57: 717-745.

Butlin R. K., Ritchie M. G. 1994. *Mating behaviour and speciation*. In: Slater P. J. B., Halliday T. R., editors. Behaviour and Evolution: Cambridge University Press. p. 43-79.

Byrne B. C. 1999. *Behaviour-genetic analysis of lovesongs in desert species of Drosophila*. [Ph.D.]. [Leicester]: University of Leicester.

Cobb M., Burnet B., Connolly K. 1988. Sexual isolation and courtship behavior in *Drosophila simulans*, *D. mauritiana* and their interspecific hybrids. *Behav Genet* 18: 211-255.

Costa C. T. A., Kuhn G. C. S., Sene F. M. 2000. Low courtship song variation in South and Southeastern Brazilian populations of *Drosophila meridio- nalis* (Diptera, Drosophilidae). *Rev Bras Biol* 60: 53-61.

Costa C. T. A., Sene F. M. 2002. Characterization of courtship sounds of species of the subgroup *fasciola* (Diptera, Drosophilidae, *Drosophila repleta* group): Interspecific and interpopulational analyses. *Brazil J Ge- net* 62: 573-583.

Cowling D. E., Burnet B. 1981. Courtship songs and genetic control of their acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. *Anim Behav* 29: 924-935.

Coyne J. A., Orr H. A. 2004. *Speciation.* Sunderland, MA: Sinauer.

de Brito R. A., Manfrin M. H., Sene F. M. 2002. Mitochondrial DNA phylogeography of Brazilian populations of *Drosophila buzzatii*. *Genet Mol Biol* 25: 161-171.

Durando C. M., Baker R. H., Etges W. J., Heed W. B., Wasserman M., de Salle R 2000. Phylogenetic analysis of the *repleta* species group of the genus *Drosophila* using multiple sources of characters. *Mol Phylogenet Evol* 16: 296-307.

Etges W. J. 2002. Divergence in mate choice systems: does evolution play by rules? *Genetica* 116: 151-166.

Etges W. J., Noor M. A. F. 2002. In. *Genetics of mate choice: From sexual selection to sexual isolation.* New York: Kluwer. p. 260.

Etges W. J., Oliveira C. C., Gragg E., Ortiz-Barrientos D., Noor M. A. F., Ritchie M. G. 2007. Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype x environment interactions. *Evolution* 61: 1106-1119.

Etges W. J., Over K. F., Oliveira C. C., Ritchie M. G. 2006. Inheritance of courtship song variation among geographically isolated populations of *Drosophila mojavensis*. *Anim Behav* 71: 1205-1214.

Ewing A. W. 1983. Functional aspects of *Drosophila* courtship. *Biol Rev* 58: 275-292.

Ewing A. W. 1989. *Arthropod Bioacoustics: Neurobiology and Behaviour.* Edinburgh, Scotland: Edinburgh University Press.

Ewing A. W., Miyan J. A. 1986. Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. *Anim Behav* 34: 421-429.

Franco F. F., Silva-Bernardi E. C. C., Sene F. M., Hasson E. R., Manfrin M. H. 2010. Intra- and interspecific divergence in the nuclear sequences of the clock gene *period* in species of the *Drosophila buzzatii* cluster. *J Zool Syst Evol Res* 48: 322-331.

Gleason J. M., Ritchie M. G. 1998. Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: Do sexual signals diverge the most quickly? *Evolution* 52: 1493-1500.

Grace J. L., Shaw K. L. 2012. Incipient sexual isolation in *Laupala cerasina*: Females discriminate population-level divergence in acoustic characters. *Curr Zool* 58: 416−425.

Henry C. S., Brooks S. J., Duelli P., Johnson J. B., Wells M. M., Mochizuki A. 2012. Parallel evolution in courtship songs of North American and European green lacewings (Neuroptera: Chrysopidae). *Biol J Linn Soc* 105: 776-796.

Henry C. S., Wells M. L. M., Holsinger K. E. 2002. The inheritance of mating songs in two cryptic, sibling lacewing species (Neuroptera: Chrysopidae: *Chrysoperla*). *Genetica* 116: 269-289.

Henry C. S., Wells M. L. M., Simon C. M. 1999. Convergent evolution of courtship songs among cryptic species of the *carnea* group of green lacewings (Neuroptera: Chrysopidae: *Chrysoperla*). *Evolution* 53: 1165-1179.

Hoikkala A. 2005. *Inheritance of male sound characteristics in Drosophila species.* In: Drosopoulos S, Claridge MF, editors. Insect sounds and communication: Physiology, behaviour, ecology and evolution. *Boca Ra- ton:* CRC Taylor & Francis. p. 167-177.

Hoikkala A., Kaneshiro K. Y., Hoy R. R. 1994. Courtship songs of the picture-winged *Drosophila planitibia* subgroup species. *Anim Behav* 47: 1363-1374.

Hoy R. R., Hoikkala A., Kaneshiro K. 1988. Hawaiian courtship songs: Evolutionary innovation in communication signals of *Drosophila*. *Science* 240: 217-219.

Kokudai C. B. S., Sene F. M., Manfrin M. H. 2011. Sympatry and asymmetric introgression between the cactophilic species *Drosophila serido* and *Drosophila antonietae* (Diptera: Drosophilidae). *Ann Entomol Soc Am* 104: 434-442.

Kusmierski R., Borgia G., Uy A., Crozier R. H. 1997. Labile evolution of display traits in bowerbirds indicates reduced effects of phylogenetic constraint. *Proc R Soc Biol Sci Ser* B 264: 307-313.

Kyriacou C. P., Hall J. C. 1980. Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc Natl Acad Sci USA* 77: 6729-6733.

Kyriacou C. P., Hall J. C. 1982. The function of courtship song rhythms in *Drosophila*. *Anim Behav* 30: 794-801.

Laurin M., Canoville A., Quilhac A. 2009. Use of paleontological and molecu- lar data in supertrees for comparative studies: the example of lissamphibi- an femoral microanatomy. *J Anat* 215: 110-123.

Liimatainen J., Hoikkala A., Aspi J., Welbergen P. 1992. Courtship in *Drosophila montana*: the effects of male auditory signals on the behaviour of flies. *Anim Behav* 43: 35-48.

Maddison W. P., Maddison D. R. 2010. *Mesquite: A modular system for evo- lutionary analysis.* Version 2.74.

Manfrin M. H., de Brito R. O. A., Sene F. M. 2001. Systematics and evolution of the *Drosophila buzzatii* (Diptera: Drosophilidae) cluster using mtDNA. *Ann Entomol Soc Am* 94: 333-346.

Manfrin M. H., Sene F. M. 2006. Cactophilic *Drosophila* in South America: A model for evolutionary studies. *Genetica* 126: 57-75.

Mendelson T. C., Shaw K. L. 2005. Sexual behaviour: Rapid speciation in an arthropod. *Nature* 433: 375-376.

Mendelson T. C., Shaw K. L. 2012. The (mis)concept of species recognition. *Trends Ecol Evol* 427: 421-427.

Moraes E. M., Spressola V. L., Prado P. R. R., Costa L. F., Sene F. M. 2004. Divergence in wing morphology among sibling species of the *Drosophila buzzatii* cluster. *J Zool Syst Evol Res* 42: 154-158.

Moraes M. A. M. 1992. *Isolamento sexual entre populações da superespécie Drosophila serido* (Diptera, Drosophilidae). [M. S.]. [São Paulo]: Universidade de São Paulo.

Noor M. A. F., Williams M. A., Alvarez D., Ruiz-Garcia M. 2000. Lack of evolutionary divergence in courtship songs of *Drosophila pseudoobscura* subspecies. *J Insect Behav* 13: 255-262.

Oliveira C. C., Manfrin M. H., Sene F. M., Jackson L. L., Etges W. J. 2011. Variations on a theme: diversification of cuticular hydrocarbons in a clade of cactophilic *Drosophila*. *BMC Evol Biol* 11:179.

Oliveira D. C. S. G., Almeida F. C., O'Grady P., Armella M. A., de Salle R., Etges W. J. 2012. Monophyly, divergence times, and evolution of host plant use inferred from a revised phylogeny of the *Drosophila repleta* species group. *Mol Phylogenet Evol* 427: 421-427.

Panhuis T. M., Butlin R., Zuk M., Tregenza T. 2001. Sexual selection and speciation. *Trends Ecol Evol* 16: 364-371.

Price J. J., Lanyon S. M. 2002. Reconstructing the evolution of complex bird song in the oropendolas. *Evolution* 56: 1514-1529.

Raven. 2003. ver. Pro 1.3. Version Pro 1.3. Ithaca, New York, USA. www.birds.cornell edu/ raven: Cornell Laboratory of Ornithology.

Reeve J., Abouheif E. 2003. *Phylogenetic Independence.* Version 2.0.

Ritchie M. G., Gleason J. M. 1995. Rapid evolution of courtship song pattern in *Drosophila willistoni* sibling species. *J Evol Biol* 8: 463-479.

Ritchie M. G., Halsey E. J., Gleason J. M. 1999. *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song. *Anim Behav* 58: 649-657.

Ritchie M. G., Saarikettu M., Hoikkala A. 2005. Variation, but no covariance, in female preference functions and male song in a natural population of *Drosophila montana*. *Anim Behav* 70: 849-854.

Ritchie M. G., Saarikettu M., Livingstone S., Hoikkala A. 2001. Characteriza- tion of female preference functions for *Drosophila montana* courtship song and a test of the temperature coupling hypothesis. *Evolution* 55: 721-727.

Ritchie M. G., Townhill R. M., Hoikkala A. 1998. Female preference for fly song: playback experiments confirm the targets of sexual selection. *Anim Behav* 56: 713-717.

Rodriguez-Trelles F., Alarcón L., Fontdevila A. 2000. Molecular Evolution and phylogeny of the buzzatii complex (*Drosophila repleta* group): A maximum-likelihood approach. *Mol Biol Evol* 17: 1112-1122.

Ruiz A., Cansian A. M., Kuhn G. C. S., Alves M. A. R., Sene F. M. 2000. The *Drosophila serido* speciation puzzle: putting new pieces together. *Genetica* 108: 217-227.

Ruiz A-, Ranz J. M., Cáceres M., Segarra C., Navarro A., Barbadilla A. 1997. Chromosomal evolution and comparative gene mapping in the *Drosophila repleta* species group. *Brazil J Genet* 20: 553-565.

Ruiz A., Wasserman M. 1993. Evolutionary cytogenetics of the *Drosophila buzzatii* species complex. *Heredity* 70: 582-596.

Saarikettu M., Liimatainen J. O., Hoikkala A. 2005. The role of male courtship song in species recognition in *Drosophila montana*. *Behav Genet* 35: 257-263.

SAS Institute Inc. 2004. *SAS/STAT 9.1.2.* Cary, NC: SAS Institute, Inc.

Sene F. M., Manfrin M. H. 1998. A chamber to record the courtship sound in *Drosophila*. *Dros Inf Serv* 81: 203-204.

Sene F. M., Pereira M. A. Q. R., Vilela C. R. 1982. *Evolutionary aspects of cactus breeding* Drosophila in South America. In: Barker J. S. F., Starmer W. T., editors. Ecological Genetics and Evolution. The Cactus-Yeast-*Drosophila* Model System. Sydney: Academic Press. p. 97–106.

Silva A. F. G., Sene F. M. 1991. Morphological geographic variability in *Drosophila serido* (Diptera, Drosophilidae). *Rev Bras Entomol* 35: 455-468.

Sony. 2006. *Sound Forge 7.0.* Sony Creative Software Inc., Madison, Wiscon- sin, USA http://www.sonycreativesoftware.com/.

Swofford D. L. 2000. *PAUP**. Version 4.0. Sunderland, MA: Sinauer.

Symonds M. R. E., Elgar M. A. 2004. The mode of pheromone evolution: evidence from bark beetles. *Proc R Soc Lond B Biol Sci* 271: 839-846.

Throckmorton L. H. 1982. Pathways of evolution in the genus *Drosophila* and the founding of the *repleta* group. In: Barker J. S. F., Starmer W. T., editors. *Ecological genetics and evolution.* Sydney, Australia: Academic Press. p. 32-47.

Tomaru M., Matsubayashi H., Oguma Y. 1995. Heterospecific inter-pulse intervals of courtship songs elicit female rejection in *Drosophila biauraria*. *Anim Behav* 50: 905-914.

Tomaru M., Oguma Y. 1994. Genetic basis and evolution of species-specific courtship song in the *Drosophila auraria* complex. *Genetic Research,* Cambridge 63: 11-17.

Vilela C. R. 1983. A revision of the *Drosophila repleta* species group (Diptera: Drosophilidae). *Rev Bras Entomol* 27: 1-114.

von Schilcher F. 1976. The role of auditory stimuli in the courtship of *Drosophila melanogaster*. *Anim Behav* 24: 18-26.

Wasserman M. 1992. Cytological evolution of the *Drosophila repleta* species group. In: Krimbas C. B., Powell J. R., editors. *Drosophila* Inversion Polymorphism. *Boca Raton:* CRC Press. p. 455-552.

Yamada H., Sakai T., Tomaru M., Doi M., Matsuda M., Oguma Y. 2002. Search for species-specific mating signal in courtship songs of sympatric sibling species, *Drosophila ananassae* and *D. pallidosa*. *Genes Genet Syst* 77: 97-106.

P. K.