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MALE COURTSHIP SONG AND FEMALE PREFERENCE VARIATION BETWEEN PHYLOGEOGRAPHICALLY DISTINCT POPULATIONS OF *DROSOPHILA MONTANA*

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Understanding the variation within and between populations in important male mating traits and female preferences is crucial to theories concerning the origin of sexual isolation by coevolution or other processes. There have been surprisingly few studies on the extent of variation and covariation within and between populations, especially where the evolutionary relationships between populations are understood. Here we examine variation in female preferences and a sexually selected male song trait, the carrier frequency of the song, within and between populations from different phylogeographic clusters of *Drosophila montana.* Song is obligatory for successful mating in this species, and both playback and field studies implicate song carrier frequency as the most important parameter in male song. Carrier frequency varied among three recently collected populations from Oulanka (Finland), Vancouver (Canada), and Colorado (central United States), which represent the main phylogeographic groups in *D. montana.* Males from Colorado had the most distinct song frequency, which did not follow patterns of genetic differentiation. There was considerable variation in preference functions within, and some variation between, populations. Surprisingly, females from three lines from Colorado seem to have preferences disfavoring the extreme male trait found in this population. We discuss sources of selection on male song and female preference.

KEY WORDS: **Female preference functions, male courtship song, mate choice, population variation, sexual selection, speciation.**

The extent of variation in male mating traits and female preference functions is poorly studied, yet it is vitally important for understanding sexual selection and sexual isolation (e.g., Gavrilets et al. 2001). If there is sufficient variation, assortative mating is expected to generate a positive covariance between important male mating traits and female preferences within populations, and coevolution between these traits can lead to the divergence of mate recognition systems between populations and thereby cause sexual isolation and speciation (Lande 1981, 1982). Much of the research on signal-preference variation in natural systems

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has focused on the extent of variance and covariance between these traits, especially the genetic covariance within populations. Models have identified its magnitude as a key to understanding both the likelihood of runaway and condition-indicator mechanisms of sexual selection (Arnold 1983; Andersson 1994). The existence of genetic covariance between signals and preference (and between these signals and male condition) has been demonstrated in some species (Bakker and Pomiankowski 1995; Kotiaho 2000; Tomkins et al. 2004), but not in others (Morris et al. 1996; Day and Gilburn 1997; Ritchie et al. 2005). However, there have been only a few studies on the extent of variance and covariance in male traits and female preferences between populations, especially where the evolutionary relationships between populations are understood. If the coevolution of traits can generate sexual isolation, the existence of differentiated populations with correlated variation is to be expected (preferences and traits changing "in step," with different populations being distributed along a "line of equilibrium," Lande 1981), but is not inevitable. For example, if direct selection acts strongly on a male trait in some populations, covariance within a population need not necessarily be reflected by strong covariation between mean male trait and preference among populations. Direct selection on either trait or preference can severely reduce the likelihood of runaway coevolution (e.g., Hall et al. 2000)

Positive covariation among populations is surprisingly scarcely reported. As examples of coordination, Zuarth and Macías Garcia (2006) found positive assortative mating preferences and variation in male sexual morphology between five populations of a Mexican fish. Tilley et al. (1990) found sexual isolation covaried with geography in a salamander species, and between population variation in male song of the brown planthopper is matched by similar variation in female preferences (Butlin 1996), but on the whole geographic variation is rarely studied for both mating signals and female preferences (Endler and Houde 1995; Ritchie 1996). Simmons et al. (2001) found a lack of coordinated geographic variation between male song and female preference functions for song parameters in crickets (see also Gray and Cade 2000). Hamilton and Poulin (1999) also failed to find covariation between male nuptial coloration and female preference in different populations of a New Zealand freshwater fish.

Noncoevolutionary models of signal-preference evolution have also been proposed. Sensory exploitation models of trait evolution rely on female preferences evolving in a nonsexual context and being subsequently exploited by male traits shifting into the range of the female biases (for reviews see Shaw 1995; Rodriguez and Snedden 2004). Sensory drive can lead to coordinated variation among populations: Boughman (2001) found that sexual isolation occurred between populations of sticklebacks because female spectral sensitivities were tuned to local environmental conditions and male traits were tuned to the local female sensitivities. Antagonistic sexual coevolution models emphasize that male traits evolve to overcome female resistance to mating, so the traits will not necessarily be tightly coordinated. Antagonistic sexual conflict will give rise to a dynamic interaction between male coercion and female resistance which may behave unpredictably (Gavrilets 2000; Gavrilets et al. 2001; Arnqvist 2006). Empirical studies of systems suggesting conflict over the outcome of mating indicate that, despite a broad pattern of covariance between female and male traits between species, interactions can be "out of step" when one gender happens to have the upper hand (see also Bergsten et al. 2001; Arnqvist and Rowe 2002).

Here we examine the variation in the carrier frequency of male song, a trait which is known to be a target of sexual selection, and female preference function for this trait within and between populations of *Drosophila montana*. We sampled recently collected populations from the main phylogeographic groupings of *D. montana*, from Finland, Canada, and the central United States. *Drosophila montana* belongs to the *virilis* species group, which can be divided into two subgroups, the *virilis* clade containing *D. virilis*, *D. lummei*, *D. americana americana*, *D. americana texana*, and *D. novamexicana*, and the generally genetically and phenotypically more diverse *montana* clade (*D. kanekoi*, *D. ezoana*, *D. littoralis*, *D. borealis*, *D. flavomontana,* and *D. montana*). Species of the *montana* clade are mostly found north of latitude 40°N and the main distribution of *D. montana* comprises Scandinavia, the Rocky Mountains in North America, Canada, and Japan (Throckmorton 1982). A recent phylogeographic study examined mitochondrial DNA (mtDNA) and microsatellite loci and found considerable population structure within the northern hemisphere. Finnish, North American, and Japanese populations form different genotypic clusters thought to represent different radiations from refugia within the past one million years. There was also evidence for substructure within the Finnish and North American radiations (Mirol et al., 2007).

The mating system of *D. montana* is dominated by male courtship song and female preferences. The song, produced by wing vibration, is obligatory for successful mating (Liimatainen et al. 1992) and female preference can be measured by playback experiments with synthetic song (Ritchie et al. 1998; Ritchie et al. 2001). Both playback and field studies implicate song carrier frequency as the most important target of female preference (Aspi and Hoikkala 1995), although other song components can also influence sexual selection and/or species recognition (Saarikettu et al. 2005). Carrier frequency probably functions as an indicator of male condition, as the offspring of males with high frequency song have higher viability (Hoikkala et al. 1998). In this study we analyzed male courtship song frequency and female preference variation for this trait in recently collected samples from the main phylogeographic clades of *D. montana*, from Finland, Canada, and the central United States.

Methods

COLLECTIONS

Isofemale lines (from here on abbreviated as "lines") were established from collections made in 2003 in Oulanka, Finland (66°25′N, 29°0′E), in Vancouver and surroundings in British Columbia in Canada (49°16′N, 122°55′W), and in Crested Butte, Colorado (38°52′N, 106°59′W). We collected 23 lines from Canada, seven from Colorado, and 16 from Oulanka. Some perished during the first few generations in the lab, thus a different number of lines were available for the different experiments. Even though sampling expeditions to other geographical regions have been made (northern parts of China, around Moscow and eastern Canada, and Yorkshire and East Scotland in the United Kingdom), no other *D. montana* populations were found.

Lines were maintained in multiple vials with approximately 50 flies per vial on malt medium, sprinkled with live yeast. They were housed at a constant temperature of 19°C (with an average variation ± 0.5°C and a fluctuation 0.1°C) and a 24 h light regime, simulating natural conditions during the mating season. Emerging flies were sexed under light CO_2 anesthesia and separated into single-sex vials within three days after emergence to ensure virginity. After becoming sexually mature at the age of three weeks, males and females were isolated individually in vials shortly before being used for playback experiments or song recording.

EXPERIMENTS

Male song

Males were stimulated to produce courtship song by placing them with a mature virgin female of the same strain into a small net covered petri dish. Song was recorded with an AKG C 1000 S microphone and a Marantz Professional tape recorder (model no. 74PDM 502/02B). Males from different populations and strains were recorded in a randomized order at a constant ambient temperature of 20°C (±1°C). Altogether we used males from nine lines from the Oulanka population, 23 lines from the Vancouver, and six lines from the Colorado population. Song traits were analyzed with the Signal 4.0 sound analysis system (Engineering Design, Belmont, MA). The carrier frequency was taken as the highest peak frequency from a fast Fourier transformation (FFT) transform within each of three separate pulse trains whenever available. The songs of approximately three F1 males per line were recorded (mean 2.89 ± 0.64 SD) and the average of three pulse trains for each male was used as the unit of analysis in a fully nested analysis of variance (ANOVA). The frequency data were normally distributed and thus used untransformed (One-sample Kolmogorov–Smirnov test, $N = 115$ males, $Z = 0.894$, $P = 0.4$).

Female preference

Female preference functions were analyzed in generations F_3 to F_8. In *D. montana,* female preference for song can be assessed through a female wing lifting gesture (e.g., Vuoristo et al. 1996). Wing lifting signals the female's acceptance and is usually followed by the mounting of the male and, almost invariably, followed by copulation. Synthetic song can be used to test female preference for particular song traits (Ritchie et al. 1998). We synthesized courtship song with a custom made computer program within the SIGNAL software (Electronic Design, Belmont, MA), with which we generated sine waves of the desired length and amplitude. The carrier frequency of song was manipulated, whereas all other parameters were held constant (pulse length [PL] 12 ms, with a 3 ms rise and decline, interpulse interval [IPI] 50 ms), providing us with seven different models of synthetic song carrier frequency (FRQ: 200, 225, 250, 275, 300, 325, or 350 Hz), which covered the range of songs produced naturally by males. Although IPI has been shown to be involved mainly in species recognition processes and to be under stabilizing selection (Hoikkala et al. 2005), previous studies of female preference in Finnish *D. montana* have shown that within this range females exert directional selection favoring higher carrier frequencies (see fig. 1 in Ritchie et al. 1998; Ritchie et al. 2001).

For each preference test, a mature virgin female was aspirated into a Perspex cylinder with a nylon net bottom and top (1.5 cm diameter, 1 cm high). The mean age of experimental females was 22 days ± 1.94 SD. A male with amputated wings (wingless males of the *virilis* species group do not produce sounds, as has been shown by experiments using a velocity sensitive microphone that would pick up sounds produced by vibration, A. Hoikkala, pers. comm.) provided an additional stimulus which enhances female response without changing the shape of the preference function (Ritchie et al. 1998). The male was dewinged under a slight CO_2 anesthesia at least one day prior to its use. A tray holding seven cylinders was placed 5 cm above the membrane of a loudspeaker (subwoofer of a MicroMedia PC sound system from Boston Acoustics Inc., Peabody, MA), resulting in a 98-dB amplitude of the songs at each chamber, standardized for each playback using a Realistic 33−2050 sound level meter.

We conducted the playback experiments in three blocks and each female was presented with three or four different song models. Usually, only one song per day was played to each female; however, occasionally a few females were tested twice on the same day with a break of at least 8 h between tests. Each female was tested on a maximum of four successive days. The temperature was held constant between 18 and 21°C. The position of an individual female's chamber on the tray was altered for each of the tests and the songs were played back in a random order to each group. Every song model was played for 10 min and the occurrence of the wing lifting posture of each female was recorded. Flies were handled without anesthesia on experimental days.

Because of the binary nature of the female response, preference variation was analyzed using a generalized linear model

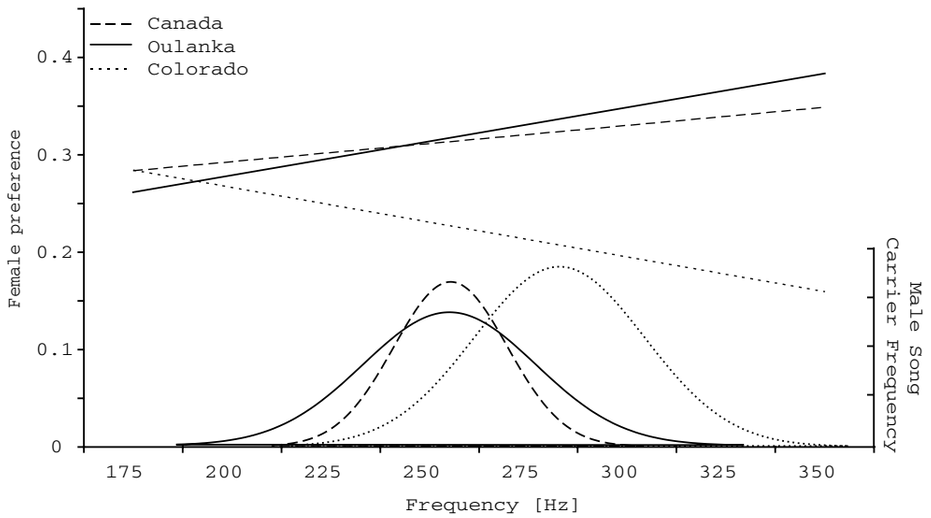

Figure 1. Top lines: percentage of females responding to synthetic song differing in carrier frequency. Not all females were tested with all playbacks; therefore, sample sizes vary among playbacks from $N = 119$ to 122 females from 18 isofemale lines for the Vancouver (----), $N = 33$ to 46 females from three lines for the Colorado (·····) and $N = 58$ to 108 females from ten lines for the Oulanka (——) population. The linear preference functions were obtained by regression, with $R^2 = 0.11$ for Vancouver females ($\beta = 0.01$), $R^2 = 0.58$ for females from Oulanka ($\beta = 0.02$), and $R^2 = 0.31$ for Colorado females ($\beta = -0.02$). Bottom curves: fitted normal distribution of male song carrier frequency in percent for individual males (rather than line means) from the Vancouver (---- $N = 68$), Oulanka (—— $N = 28$) and Colorado (····· N = 19 males) populations.

of binomial proportions of female responses. The model included terms for EXPERIMENTAL BLOCK (as a random term), FREQUENCY and POPULATION and their INTERACTION as well as LINES within POPULATIONS and FEMALES within LINE (the latter were nested terms), using Genstat5 (1993). Previous studies of the Finnish population have shown that preference is linear over the natural range of frequencies used here (Ritchie et al. 2001); however, we included a quadratic term for the FREQUENCY term and the interaction term for FREQUENCY × POPULATION in case this differed between strains. The significance of terms was assessed using analyses of deviation from fitted models. For exact sample sizes for female preference analysis see legend for Figure 1.

Results

MALE SONG

A fully nested ANOVA (Table 1) showed that most variation in song frequency (55%) was among MALES within LINES. There was significant variation among POPULATIONS, with a variance component of around 9%. The appropriate corresponding probability value is debatable. Tested with the LINE within POPULATION denominator MS the probability is 0.013. However, because of the nonsignificant LINE component, a pooled estimate of variance from the LINE and MALE terms can be used (following the procedure of Sokal and Rohlf 1981, Box 10.2), which results in a probability value of 0.005. Colorado males produced a courtship song with a significantly higher carrier frequency than males from the other populations (Fig. 1, posthoc Tukeys HSD, mean difference (±SE) to Vancouver 21.16 ± 7.86, $P = 0.029$, to Oulanka 23.25 ± 9.04, $P = 0.038$).

FEMALE PREFERENCE

Table 2 presents the analysis of preference variation. Female responsiveness differed between experimental blocks. The nonsignificant quadratic terms show that females do not exert any nonlinear selection on carrier frequency. Across all the data, most

Table 1. Results of a nested analysis of variance on carrier frequency of male courtship song, with males nested within lines and lines nested within populations. Values in brackets are based on a pooled estimate of variance from the line and male terms.

	df	MS	Percentage of total variance	*F*	*P*
Population	2	11541.7	8.81	4.79	0.013
				(5.46)	(0.005)
Line within population	43	2408.1	5.97	1.24	0.21
Male within line	74	1944.8	55.57	6.62	<0.001
(Pooled within population)	117	2115.1			
Error	240	293.7			

Table 2. Results from a general linear model with logit link function on female response to song playbacks with different carrier frequencies. *P*-values are based on the mean deviance which is approximately distributed as χ^2.

	df	Deviance	Mean deviance	*P*
Experimental block	2	9.531	4.765	0.009
Preference for frequency (linear)	1	2.290	2.290	0.130
Preference for frequency (quadratic)	1	1.463	1.463	0.226
Responsiveness among populations	2	12.675	6.337	0.002
Responsiveness among lines within populations	28	94.056	3.359	<0.001
Responsiveness among females within lines within populations	437	705.635	1.615	<0.001
Preference for frequency × populations (linear)	2	8.620	4.310	0.013
Preference for frequency × populations (quadratic)	2	1.263	0.631	0.532
Total	1623	2005.300		

of the variation was among individual females and between lines within populations, although there was also significant variation in both responsiveness and the shape (slope) of the preference function for frequency among populations (Table 2). Canadian and Finnish females overall preferred song with high carrier frequencies (regression coefficient from individual female fits on frequency, Canada $\beta = 0.073$, $P = 0.003$; Oulanka $\beta = 0.140$, $P < 0.001$), as expected from previous studies (e.g., Ritchie et al. 2001), but females from the Colorado overall favored songs with low frequencies ($\beta = -0.226$, $P < 0.001$, Fig. 1). For illustrative purposes, Figure 2 shows the fitted lines from separate models for each population including the terms FREQUENCY and LINES × FREQUENCY. This demonstrates the shape of the linear selection gradient for each line within each population, and demonstrates the extreme variation among lines within populations; all populations contain some lines showing "reversed" preference functions. There was also substantial variation in responsiveness (the overall percentage of females responding to playbacks) among populations, and among lines and individual females. Colorado females were the most discriminating overall: Canadian and Finnish females produced the wing lifting gesture in 33% (± 2% SD) and 34% (± 2%) of the song playbacks, respectively, the Colorado females answered only to 21% (± 3%) of the songs.

Discussion

We show here that there is substantial variation in female song preference and also in an important component of male song within and between geographically and phylogenetically distinct populations of *D. montana*. Males from Colorado produced a courtship song with a significantly higher carrier frequency than males from either the Canadian or the Finnish population (also see Routtu et al., in press). Female preference variation is more complex. There is substantial variation within populations, but also variation in both the level of responsiveness and the shape of the preference function among populations. Interestingly, females from Colorado do not have an especially strong preference for high carrier frequency. If anything, they prefer courtship songs with low carrier frequencies, whereas females from the other populations overall prefer high frequency song. Females from Colorado were also more discriminating (responded less often) than females from Canada and Finland.

Previous studies of *D. montana* have shown that females prefer males with higher song frequency, but these females have all been from Finland. Sexual selection in the field favors males producing short and dense (i.e., high frequency) sound pulses (Aspi and Hoikkala 1995) and playback experiments have shown that the most successful song has high frequency and short pulse length (Ritchie et al. 1998). The preference function for song frequency is stabilizing but peaks outside of the upper limit of the range of the male trait, hence causing effective directional selection (Ritchie et al. 2001). Carrier frequency is a potential indicator of male quality because high frequencies were associated with enhanced offspring survival (Hoikkala et al. 1998; Hoikkala and Suvanto 1999). Our data demonstrate substantial variation in preferences exists within all populations. Overall there is positive directional selection on frequency in the Finnish and Canadian samples, but the preference function favoring high frequency is less strong or even absent in female flies from Colorado. This pattern of most differentiation between Colorado and the other populations does not coincide with the genetic differentiation of the populations. Colorado samples are differentiated at microsatellite loci, but with respect to mtDNA they are very similar to the Vancouver samples (Mirol et al. 2007). Morphological and other song traits also show distinctive patterns of differentiation among these three populations, suggesting selection has shaped distinct patterns of variation in each clade beyond a simple increase in phenotypic divergence with phylogenetic history (Routtu et al., in press).

Although we have sampled only three populations song carrier frequency and preference do not seem to be coevolving in the straightforward manner predicted by some models of

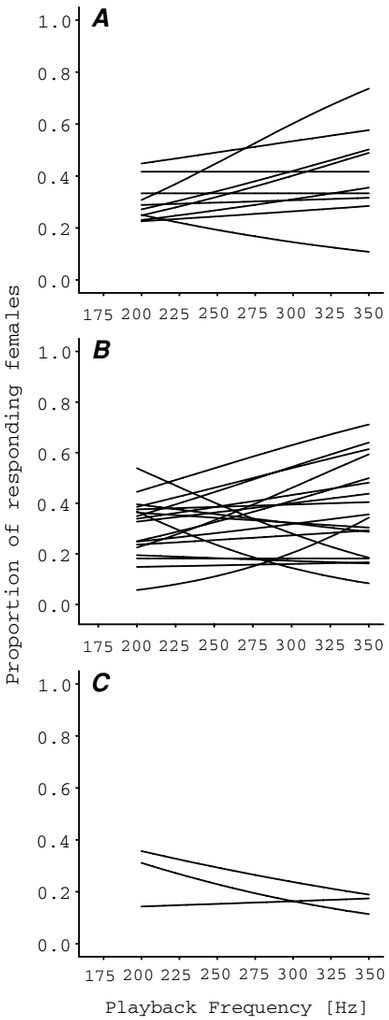

Figure 2. Linear selection gradients for each line within each population: (A) Oulanka, Finland, (B) Vancouver, Canada, (C) Colorado, United States. Lines from separate models were fitted for each population, including the terms frequency and lines × frequency only.

signal-preference coevolution (e.g., Lande 1981; Arnold 1983). Between clade divergence is probably not evolving by a Fisherian process and it seems that the quality indicator process implied in Finnish samples is unlikely to apply in all populations. Some ecological conditions may favor an indicator process, whereas others favor the Fisher process (e.g., ecological stability is thought to influence the strength of trait-preference genetic covariation in seaweed flies, Gilburn and Day 1994; Day and Gilburn 1997). Within one Finnish population of *D. montana*, there is no detectable genetic correlation between trait and preference (Ritchie et al. 2005), so the traits can evolve independently (to the extent that they do not mutually influence each other). An indicator process could still apply to the Colorado population if the direction of the correlation between frequency and fitness had changed, or another trait had become a more reliable indicator (different traits that are under selection by female preference might indicate different aspects of quality, the most important of which varies with ecology [Pomiankowski and Iwasa 1993; Johnstone 1995]). Studies of multivariate geographic variation in preference functions are important but scarce (e.g., Brooks and Endler 2001; Blows et al. 2003).

Different sources of direct selection on song and/or preference could arise as adaptations to different environments. For example, background noise frequencies and levels in different populations might have led to the shift in carrier frequency and associated preferences. The Colorado population inhabits high altitudes, as opposed to the other two populations from near sea level. Environmental selection has previously been shown to lead to different coevolutionary dynamics in different environments. In guppies, female preference for male coloration was correlated with the water color in different populations (Endler and Houde 1995; for an example on sticklebacks, see also Boughman 2001). The divergence of mate recognition signals between two subspecies of the cricket frog was influenced by the effect of their local habitats on transmission efficiency (Ryan 1990) and the light environment selects on color signals and opsin genes in cichlid fish (Terai et al. 2006). Peculiarities of the Colorado habitat (where flies were collected at 3000 m altitude) might also have an influence on male song. A higher wing load (i.e., body weight to wing area) is required to fly in low air pressures, thus natural selection for effective flight performance at high altitudes might not only affect wing beat amplitude, as shown for hymenopterans (Dillon et al. 2006), but might also affect wing beat rate and, in turn, indirectly song. The local parasitic fauna can also influence song or preference evolution (Zuk and Kolluru 1998). The attraction of parasitic flies to song can influence song parameters and phonotactic behavior in crickets and is thought to influence the lack of coordination in song and preference variation in *Teleogryllus oceanicus* (Simmons et al. 2001). Increased costs of preferences are thought to underlie an example of a negative between population correlation between trait and preference for male size in *Xiphophorus* (Morris et al. 1996).

Our results could also be influenced by antagonistic coevolution. Sexual conflict models do not make precise predictions about the outcome of the evolutionary arms race between male coercion tactics and female resistance, although overall some coordination is expected (Gavrilets et al. 2001). It is unclear if and how sexual selection and sexual conflict coexist within a species (for a review see Tregenza et al. 2006). In *D. melanogaster*, mating alters life history to the detriment of females (Rice 1996; Wigby and Chapman 2005) and evolving a different preference function could be one way of increasing female resistance to stimulating male traits (Gavrilets et al. 2001). Such an interpretation of the

pattern found here might be favored by the especially high carrier frequency of Colorado males. These might have made Colorado males extremely attractive (or manipulative), to which females responded by changing the direction of their preference functions as a way to resist this exploitation/manipulation. Our finding that Colorado females are highly discriminating perhaps is consistent with this idea. However, reconstructing the evolution of song and preference variation in *D montana* would require the analysis of more populations and improved sampling in the Colorado region.

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