Widespread genetic linkage of mating signals and preferences in the Hawaiian cricket Laupala

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The evolution of novel sexual communication systems is integral to the process of speciation, as it discourages gene flow between incipient species. Physical linkage between genes underlying male–female communication (i.e. sexual signals and preferences for them) facilitates both rapid and coordinated divergence of sexual communication systems between populations and reduces recombination in the face of occasional hybridization between diverging populations. Despite these ramifications of the genetic architecture of sexual communication for sexual selection and speciation, few studies have examined this relationship empirically. Previous studies of the closely related Hawaiian crickets Laupala paranigra and Laupala kohalensis have indirectly suggested that many of the genes underlying the difference in pulse rate of male song are physically linked with genes underlying the difference in female preference for pulse rate. Using marker-assisted introgression, we moved 'slow pulse rate' alleles from L. paranigra at five known quantitative trait loci (QTL) underlying male pulse rate into the 'fast pulse rate' genetic background of L. kohalensis and assessed the effect of these loci on female preference. An astounding four out of five song QTL predicted the preferences of female fourth-generation backcrosses, providing direct evidence for the extensive genetic linkage of song and preference in one of the fastest diversifying genera currently known.

Keywords: genetic correlation; Fisherian runaway; reinforcement; linkage disequilibrium

1. INTRODUCTION

The sexual attraction of individuals to distinct physical characters and behavioural signals of conspecific mates is central to the maintenance of genetic boundaries between closely related species. Likewise, the evolution of new species is frequently tied to the evolution of novel sexual communication systems [1–5]. However, diversification of sexual signals is constrained by the need to maintain coordination between signaler and receiver [6] owing to the selective elimination of novel variants in signal or preference by stabilizing selection [7]. The evolution of sexual communication systems thus should involve concerted change in both male and female components of communication, a process critically dependent on maintaining genetic correlations between the traits [6,8].

A positive genetic correlation between independent preference and signal loci (linkage disequilibrium) may arise through assortative mating, as offspring inherit genes underlying both their mother's preference and father's signal [9–11]. However, such linkage disequilibrium is evolutionarily unstable because it depends upon strong and ongoing assortative mating. It may break down if preferences are only expressed under certain conditions (e.g. [12–15]) or if preferred mates are often lacking and thus preferences fail to be expressed [16].

Robust genetic correlations between signal and preference, favourable for speciation, are produced when the loci underlying the two traits are physically linked in the same chromosomal regions. The evolutionary consequences of such genetic architecture are twofold for sexual communication systems. First, robust genetic correlations can facilitate the coordinated evolution of signal and preference within populations through Fisherian processes, provided there is no cost to alternative preferences [9,11], favouring the rapid diversification of communication systems. Second, physical linkage prevents recombination between loci following occasional hybridization between incipient species, thus promoting both the maintenance and strengthening of behavioural barriers to reproduction once they have evolved [17–20]. Despite the important implications of physical linkage for sexual selection and speciation, little is known about its prevalence in nature. Some studies have provided varied insights about the existence of genetic correlations in different systems (e.g. [21–31]), but definitive testing is frequently constrained by a lack of available genetic tools and, further, by logistic difficulties with assessing preference functions [32].

One system in which there is apparent physical linkage between preference and signal is Laupala crickets (Orthoptera: Gryllidae). The genus has recently radiated across the Hawaiian islands, accompanied by a rapid diversification of male mating song [33,34]. Males of all species produce simple songs, consisting of a series of pulses produced via wing strokes, and females locate preferred mates based on the pulse rates of these stridulations [35,36]. Different species vary widely in both the pulse rates produced by males and the preferred pulse rates of females, and differing acoustic communication systems serve as an important barrier against hybridization between closely related species [37].
The closely related species *Laupala kohalensis* and *Laupala paranigra* are valuable models for investigating the genetics of sexual communication, as they have highly divergent pulse rates and preferences, but can be crossed in captivity to produce fertile hybrids useful for characterizing the genetics of speciation. Multiple loci are responsible for the species differences in both male pulse rate [38,39] and female pulse rate preference [40]. The genomic positions of eight quantitative trait loci (QTL) underlying male pulse rate differences between *L. paranigra* and *L. kohalensis* map to multiple locations on multiple linkage groups across the genome [39]. Recent studies revealed that two of these QTL underlying the pulse rate of males are physically linked to loci underlying the preferences of females [41,42]. In addition, a strong genetic correlation between pulse rate and preference among families of recombinant hybrid lines has provided indirect evidence that physical linkage between song and preference loci may be widespread [42]. Here, with increased power to detect preference QTL over previous work, we directly test whether each of the five largest QTL underlying male pulse rate is physically linked with a preference QTL through a marker-assisted breeding design. Replicated introgression of five independent QTL regions from slow-singing *L. paranigra* into a fast-singing *L. kohalensis* genetic background allowed us to test for resultant effects of these genomic regions on the preferences of females.

2. MATERIAL AND METHODS

(a) Breeding design

The breeding experiment generated fourth-generation backcross lines that comprised primarily the *L. kohalensis* (mean pulses per second (pps) = 3.72 ± 0.13) genetic background, but concomitantly possessed one copy of the *L. paranigra* (mean pps = 0.71 ± 0.08) allele at the focal song QTL. We used marker-assisted introgression [43], whereby individual hybrid females possessing *L. paranigra*-specific amplified fragment length polymorphism (AFLP) markers linked to the focal song QTL were repeatedly backcrossed in successive generations with males from a single inbred *L. kohalensis* line. Fourth-generation backcross offspring have an expected 96.9 per cent of their genome derived from *L. kohalensis* outside the introgressed region. See Ellison et al. [44] for more detail on the breeding design and data analysis of male songs.

We replicated the marker-assisted introgression experiments, producing three to five lines for each of the five song QTL regions. In addition, five replicate control lines were generated, whereby hybrid individuals were chosen randomly to backcross with *L. kohalensis* to produce the next generation. In the third backcross generation, multiple siblings per line were used to generate large numbers of first cousins in order to generate the large sample sizes needed for preference trials. See Ellison et al. [44] for a complete pedigree.

All crickets were reared at Cornell University in the same temperature-controlled room (constant 20°C) under identical rearing conditions (housed collectively in quart-sized glass jars as nymphs and singly in plastic specimen cups as adults). Moist Kimwipes and paper towels provided bedding. Lines were reared concurrently, such that any minor irregularity in rearing environment would have affected all lines similarly.

(b) Genotyping

We monitored each backcross line via one to five *L. paranigra*-specific AFLP markers (depending on the QTL region), spanning up to 14 cM and including flanking genomic regions surrounding the QTL. Only hybrid individuals that possessed *L. paranigra* markers across the QTL region were chosen for subsequent backcrossing to ensure preservation of the entire *L. paranigra* QTL region. The fourth backcross generation contained a mixture of siblings that either possessed or lacked *L. paranigra* alleles across the introgressed region, as well as recombinants that possessed some *L. paranigra* alleles in the QTL region. For a given line, recombinants were determined to either possess or lack the *L. paranigra* song QTL in the final analysis based on their allelic state at the marker closest to the estimated QTL location in Shaw et al. [39]. Markers used for selection and final scoring and their genomic locations are described in Ellison et al. [44].

Protocols for DNA extraction and the AFLP reactions have been described previously [44]. In generations preceding the fourth backcross, we removed one hind-leg per individual for DNA analysis without causing the death of crickets needed for crosses. Genotyping was carried out without knowledge of each female's estimated preference.

Because, by chance, song QTL persisted in some lines in which they were not selected, we screened all fourth-generation backcrosses for markers associated with all five QTL. The presence or absence of song QTL were included as predictor variables in a general linear mixed model of preference (measured as described below), with line replicate as a random factor.

(c) Estimating preference

To measure pulse rate preference, virgin females (three to six weeks after final moult) were given a series of choice trials featuring two stimuli. Within each trial, digitally generated songs of predetermined pulse rate were broadcast from speakers at opposite sides of a circular arena (94 cm diameter; for a diagram of the arena, see [36]). Females were offered the following pulse rates, in randomized order: 3.0 versus 3.5 pps, 3.1 versus 3.6 pps, 3.2 versus 3.7 pps, 3.3 versus 3.8 pps, 3.4 versus 3.9 pps and 3.5 versus 4.0 pps. *Laupala* females show a well-established pattern of preferring the faster rate at the slow end and the slower rate at the fast end of a range centred on their species rate [40,41]. The trials between which the female changed from preferring the faster to the slower of the two options were used to infer her preference (estimated as the midpoint of possible values). For example, if a female preferred the faster rate in the first trial above (i.e. 3.5 pps), but the slower rate in all other trials (i.e. 3.1, 3.2, 3.3 and 3.4 pps), her preference was estimated as 3.3 pps. If a female always preferred either the slower or faster option across all six trials, additional trials were carried out in the respective direction (e.g. 2.9 versus 3.4 pps or 3.6 versus 4.1 pps) until a change in preference (to the faster or slower option) was noted. Females that exhibited inconsistent choices (e.g. preferred 3.0 pps in trial one, but 3.6 pps in trial two) were assumed to be walking randomly and were excluded from the study.

Trials were conducted within a temperature-controlled (20°C), soundproof, anechoic chamber. Females were placed in the centre of the arena for acclimatization 5 min prior to the start of the trial (with sound on), and were then given 5 min to approach one of the two speakers within a region approximately 10 cm from the sound source. All trials were
carried out from 6 to 11 h after lights on, coincident with male acoustic activity. These methods have successfully quantified pulse rate preference in Laupala [41,42]. However, our estimates were more precise owing to a smaller range of pulse rates offered across the six trials and more stringent guidelines (to reduce experimental noise, females were only included in this study if they responded in at least five trials). Using the same methods, we also estimated preferences from the inbred \textit{L. kohalensis} line used for backcrossing.

3. RESULTS

We were able to score the preferences of 331 fourth-generation backcross females in accordance with our criteria. An additional 344 females did not respond in five or more trials and 141 females made inconsistent choices. These females may have been sexually unreceptive at the time of testing or may have become de-sensitized to the trial procedure prior to the completion of at least five trials. There was no apparent bias in pulse rate preferences among responding females (electronic supplementary material, figure S1 presents a distribution of all backcross females in accordance with our criteria). Moreover, 19 out of 26 female material, figure S1 presents a distribution of all backcross among responding females (electronic supplementary material, figure S1 presents a distribution of all backcross females in accordance with our criteria.

There was no apparent bias in pulse rate preferences among responding females (electronic supplementary material, figure S1 presents a distribution of all backcross females in accordance with our criteria. Furthermore, females that lacked \textit{L. paranigra} alleles at all five song QTL (including controls and some females from each of the selection lines) preferred songs closely matching \textit{L. kohalensis} (figure 1), providing evidence that our introgression procedure removed the vast majority of unknown \textit{L. paranigra} alleles influencing female preference.

Not only did the two song QTL previously found to be linked to preference (QTL 1 and 4) [41,42] significantly predict preference among responding females in the current study, but two out of the remaining three song QTL also predicted the preferred pulse rates of females (table 1 and figure 1). Only song QTL5 (located on the X chromosome) failed to predict preference, which also had the smallest effect on song among the five loci tested [44].

In all cases where song QTL also predicted preference, the direction of the effect was the same—alleles from \textit{L. paranigra} (the slower singing species) associated with females that preferred slower pulse rates. In addition, the effect sizes of each QTL on preference were generally comparable to their effects on song (table 1), with the exception of QTL2, which slowed the songs of males by approximately twice as much as it slowed the preferences of females (table 1). These song-preference associations and similar effect sizes of QTL on both traits generated a significant correlation across lines in the songs of males and the preferences of females ($r = 0.69$, $p < 0.0001$; correlation coefficient weighted by the numbers of individuals sampled in each line; see supplementary electronic material, figure S2).

Figure 1. Pulse rate preferences among (a) pure \textit{L. kohalensis} females, (b) fourth-generation backcross females with no surveyed \textit{L. paranigra} QTL, and fourth-generation backcross females with a single \textit{L. paranigra} QTL allele at (c) QTL1, (d) QTL2, (e) QTL3, (f) QTL4 and (g) QTL5. Females with multiple \textit{L. paranigra} QTL were excluded from this figure.
female preference. However, because signals have indicated a corresponding evolution of the coordinated evolution of signal and preference. Linked loci are generally similar for both traits, enabling widespread within the genome, the effect sizes of the changes of females. Not only is this linkage remarkably physically linked to genes controlling the pulse rate preference of this hypothesis and confirms that the four QTL of crosses [42] suggested extensive genomic linkage in these traits in second-generation backcrosses [23,24,30,45,46]. Strong genetic correlations between genes underlying variation in preference and signal (e.g. [26,28,29]), but it is unknown whether the co-inheritance of these loci owing to physical proximity is uninterrupted by recombination. A final group of studies showing potential support for physical linkage include cases where mutations cause abnormalities in the expression of sexual signals along with corresponding changes in the perception of, or preferences for, these signals [49–51]. Although such examples of pleiotropy represent the ultimate case of physical linkage, as the same gene influences both signal and preference, the relevance of such mutants to natural variation in signal and preference within these systems is unclear [52].

4. DISCUSSION

Few previous studies have investigated the genetic architecture of signal and signal preference in parallel and the majority have failed to detect physical linkage between genes underlying variation in preference and signal (e.g. [23,24,30,45,46]). Strong genetic correlations between pulse rate and preference in second-generation backcrosses [42] suggested extensive genomic linkage in these traits in Laupala. Our current study provides a direct test of this hypothesis and confirms that the four QTL of largest effect underlying variation in pulse rate are physically linked to genes controlling the pulse rate preferences of females. Not only is this linkage remarkably widespread within the genome, the effect sizes of the linked loci are generally similar for both traits, enabling the coordinated evolution of signal and preference.

A few experiments using artificial selection on male signals have indicated a corresponding evolution of female preference [21,22,25,31]. However, because random mating was not enforced over multiple generations in these studies, linkage disequilibrium arising from female preference (only preferring females mated with selected males) cannot be dismissed as the source of the genetic correlation [47,48]. Another group of studies demonstrate that genes underlying preference and signal are located on the same sex chromosome (e.g. [26,28,29]), but it is unknown whether the co-inheritance of these loci owing to physical proximity is uninterrupted by recombination. A final group of studies showing potential support for physical linkage include cases where mutations cause abnormalities in the expression of sexual signals along with corresponding changes in the perception of, or preferences for, these signals [49–51]. Although such examples of pleiotropy represent the ultimate case of physical linkage, as the same gene influences both signal and preference, the relevance of such mutants to natural variation in signal and preference within these systems is unclear [52].

One compelling case of co-inheritance of natural variation in signal and preference owing to physical linkage involves visual signalling in Heliconius butterflies. Assortative mating between Heliconius pachinus (yellow wings) and Heliconius cydno (white wings) is primarily based on wing coloration, which is controlled by a single Mendelian locus [27]. Male preference for female coloration is explained by the same locus, possibly reflecting pleiotropy [27]. Similar to Kronforst et al. [27], our study considers natural variation in sexual communication between two closely related species. However, Laupala differs from Heliconius in that females express preferences for male signals. This latter situation is thought to be more conducive to speciation than male-expressed preferences [53]. Furthermore, in Laupala, acoustic signal and

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**Table 1. Effect of the presence of each song QTL region on the preference of females.** The random factor ‘line’ explained 15.5% of the total variance in preference. The effect sizes of each QTL on preference were significantly different from the effect sizes on song in two cases. The interaction terms sex × QTL2 and sex × QTL5 were significant in a model with both sexes included, with the common response variable ‘pulse rate’ (QTL2: $F_{1/1303} = 5.94$, $p = 0.015$, QTL5: $F_{1/1303} = 38.6$, $p < 0.0001$). All other QTL similarly affected both traits ($p > 0.1$ for all other interactions between sex and QTL).

<table>
<thead>
<tr>
<th>QTL</th>
<th>linkage group</th>
<th>AFLP marker</th>
<th>d.f.</th>
<th>$F$</th>
<th>$p$</th>
<th>effect size (preference)</th>
<th>effect size (song)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lp1</td>
<td>PcaacB12</td>
<td>1/299</td>
<td>48.87</td>
<td>$&lt;0.0001$</td>
<td>0.239</td>
<td>0.208</td>
</tr>
<tr>
<td>2</td>
<td>Lp3</td>
<td>PgaacB65</td>
<td>1/299</td>
<td>11.06</td>
<td>0.001</td>
<td>0.106</td>
<td>0.218</td>
</tr>
<tr>
<td>3</td>
<td>Lp4</td>
<td>PtcgcB57</td>
<td>1/299</td>
<td>15.59</td>
<td>$&lt;0.0001$</td>
<td>0.161</td>
<td>0.162</td>
</tr>
<tr>
<td>4</td>
<td>Lp5</td>
<td>PgaacB5</td>
<td>1/299</td>
<td>44.36</td>
<td>$&lt;0.0001$</td>
<td>0.250</td>
<td>0.210</td>
</tr>
<tr>
<td>5</td>
<td>LX</td>
<td>PaagcB55</td>
<td>1/299</td>
<td>0.15</td>
<td>0.698</td>
<td>$-0.013$</td>
<td>0.119</td>
</tr>
</tbody>
</table>

*Data from Ellison et al. [44].

**Table 2. Phenotypes and genotype frequencies within the respective introgression lines.** All lines selected for a given QTL are included in totals. Note that additional individuals from other lines possessed the L. paranigra QTL, such that the sample sizes shown in this table are a subset of those in table 1. The Pearson’s $χ^2$-test for a deviation from the expected probability that 50% of females from fourth-generation backcrosses within each introgression line possess the L. paranigra allele under selection. ‘Without QTL’ refers to individuals homozygous for a given QTL (one L. paranigra and one L. kohalensis allele). ‘Without QTL’ refers to individuals homozygous for L. kohalensis alleles.

<table>
<thead>
<tr>
<th>QTL lines</th>
<th>AFLP marker</th>
<th>mean pulse rate preference with QTL ($μ ± σ, n$)</th>
<th>mean pulse rate preference without QTL ($μ ± σ, n$)</th>
<th>$χ^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PcaacB12</td>
<td>$3.28 ± 0.008, 23$</td>
<td>$3.55 ± 0.006, 25$</td>
<td>0.08</td>
<td>0.77</td>
</tr>
<tr>
<td>2</td>
<td>PgaacB65</td>
<td>$3.49 ± 0.010, 20$</td>
<td>$3.57 ± 0.005, 37$</td>
<td>5.07</td>
<td>0.024</td>
</tr>
<tr>
<td>3</td>
<td>PtcgcB57</td>
<td>$3.38 ± 0.012, 18$</td>
<td>$3.43 ± 0.004, 57$</td>
<td>20.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4</td>
<td>PgaacB5</td>
<td>$3.35 ± 0.008, 23$</td>
<td>$3.59 ± 0.005, 39$</td>
<td>4.13</td>
<td>0.042</td>
</tr>
<tr>
<td>5</td>
<td>PaagcB55</td>
<td>$3.66 ± 0.011, 13$</td>
<td>$3.61 ± 0.010, 17$</td>
<td>0.53</td>
<td>0.465</td>
</tr>
</tbody>
</table>

Laupala paranigra allele frequencies tended to be slightly lower among the backcrosses than the 50 per cent expected from random segregation (table 2). This deviation from random was statistically significant for three out of five QTL (table 2), and was particularly striking for QTL3, which was present in only 24 per cent of females within the QTL3 introgression lines.
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preference are quantitative, multi-genic traits. In these respects, _Laupala_ more closely exemplifies traditional models of sexual selection.

It is perhaps not by chance that the only song QTL for which we failed to detect an effect on preference resided on the X chromosome. Unlike the _L. paranigra_ alleles at the other autosomal QTLs, which are in a heterozygous state in both sexes, the allelic states at QTL5 in backcrosses differ between the sexes, with heterozygous females and hemizygous males. Without the possibility of dominance effects in males, we might anticipate that, among backcrosses, introgressed X-linked loci may have a larger impact on the songs of males than the preferences of females. Additionally, it is possible that we lacked the power to detect an effect of QTL5 on female preference, as the effect of QTL5 on male song was the smallest estimated effect in our analysis.

The degree of postzygotic isolation between _L. kohalensis_ and _L. paranigra_ is currently untested, but given the relative ease of generating fertile hybrids under laboratory conditions [38–42,44], it is probably not strong. Despite this, the genotype frequencies observed in this study raise some intriguing questions. Among fourth-generation backcross females, several of the _L. paranigra_ markers associated with female preference were less common than expected by chance (table 2). This pattern mirrors trends seen in previous generations [42], as well as those observed in males [44], suggesting that it is not because of unintentional experimental bias resulting from our preference design. One explanation for this result is that there exist _L. paranigra_ alleles linked to these QTL that have negative epistatic interactions with other genes in the _L. kohalensis_ genetic background, causing early mortality of both males and females. Physical linkage of genes underlying pre- and post-mating isolation has profound implications for the evolution of mate recognition via reinforcement [18,54]. We currently have limited insight into the implications of the observed segregation distortion, but explicit tests would be worthwhile.

One question that remains unanswered is, how does the linkage of signal and preference loci correspond to physical map location(s) of such loci? Close physical linkage of signal and preference loci may effectively eliminate the possibility of recombination or, in the extreme case, may consist of a single locus with pleiotropic effects on signal and preference. One hypothesis proposed by Alexander [55], and advocated by Hoy and co-workers [56,57], is that the central pattern generator underlying rhythmic song components in Orthoptera have common genetic components to preferences for these rhythms. Although our results are consistent with pleiotropic effects of genes influencing both traits simultaneously, it is also possible that separate song and preference loci are in close physical linkage. The introgressed regions themselves are as large as 14 cM in length and are probably often accompanied by hitchhiking flanking regions of unknown and variable length. Recombination rates (cM Mb\(^{-1}\)) vary among insects by orders of magnitude [58] and we currently lack estimates for Orthoptera. Consequently, there are certainly a large number of genes contained within the introgressed regions. Although our results are limited in providing insight into how closely positioned the loci influencing preference and song tend to be, we can conclude that these loci are sufficiently nearby to allow co-inheritance in the face of several generations of recombination. It is this co-inheritance of preference and signal that ensures their concerted evolution.

Several cases of rapid evolution of sexual signals are in response to the open-ended preferences of females with sensory biases that evolved in other contexts [59–61]. However, such rapid evolution of sexual signals is unlikely to eliminate gene flow between derived and ancestral populations, as females from both populations prefer the derived, elaborated male phenotype [62]. For sexual selection to eliminate gene flow, open-ended preferences must be sign-inverted in different populations. Little is currently known about how evolutionarily labile such biases can be, but these preferences are sometimes phylogenetically conserved [63]. Alternatively, sexual selection can curb gene flow if, as observed for pulse rate in _Laupala_, preferences are unimodal and coevolve alongside diverging sexual signals. Physical linkage between male and female components of sexual communication ensures the maintenance of the genetic correlations necessary for such coevolution [6,8,11,64]. Accordingly, the extensive linkage between loci underlying male pulse rate and female preference observed in the current study may have facilitated the extremely high rates of signal divergence and speciation observed in _Laupala_.

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