

Mutations and Natural Genetic Variation in the Courtship Song of *Drosophila*

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All *Drosophila* species have a courtship repertoire by which a male stimulates a female to mate with him. In many species, males vibrate their wings to produce courtship song, an element of courtship that plays an important role in female choice. Each species has a unique courtship song, with the major differences among species songs being in timing and/or structure. Analysis of genetic mutations has revealed 17 genes that affect courtship song in *Drosophila melanogaster*. Most of the genes were first identified as affecting another trait and were subsequently shown to affect song. Quantitative genetic studies have demonstrated a polygenic additive genetic architecture for many song traits. Few candidate genes, identified through the classical genetic approach, coincide with the regions implicated as affecting natural variation. With many new tools in genetic analysis and the multiple *Drosophila* genome projects currently underway, the ability to relate mutational and quantitative analyses will improve.

KEY WORDS: Candidate genes; courtship song; *Drosophila* species; mutations; quantitative genetics.

INTRODUCTION

The evolution of premating isolation can be a primary cause of speciation, yet the genetics underlying courtship behavior are poorly understood (Ritchie and Phillips, 1998). As an easily quantifiable behavior important to male fitness, *Drosophila* courtship song is an ideal trait for studying the genetics of mating behaviors. Courtship song is also sexually selected trait in many *Drosophila* species with a fundamental role in species recognition (e.g. Saarikettu *et al.*, 2005; Snook *et al.*, 2005).

Two fundamentally different approaches have been used to study the genetics of courtship song. The first approach uses single gene mutations to examine the genetic interactions and pathways underlying the behavior. Some of these mutations have been identified through genetic screens. Other mutations have been tested for song effects because of known effects

on other processes associated with courtship song production. Although this approach has been successful in elucidating some of the underlying neurological processes involved, many of the mutations decrease the mating success of the male. Thus, the relevance of these genes to evolution is often questionable. The second approach uses natural variation within species and differences between species to estimate the genetic architecture in terms of gene interactions (e.g. additively, dominance, epistasis), the number of genes affecting the trait, and the genetic locations of these genes. However, in this approach, the particular genes implicated have not been identified. To date, these two approaches have predominantly identified different factors, with a few exceptions, but the potential is good for combining these approaches in the future.

COURTSHIP SONG TRAITS

Courtship song is one of several behaviors contributing to the mating repertoire. The male “sings” by vibrating a wing. *D. melanogaster* males have two

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song types: pulse song and sine song. Sine song sounds like a hum and serves in acoustic priming of females prior to courtship (Kyriacou and Hall, 1984). Pulse song consists of a train of low frequency, short pulses. For *D. melanogaster*, pulse song increases male activity and decrease females activity (Bennet-Clark and Ewing, 1967; Crossley *et al.*, 1995; von Schilcher, 1976b).

Courtship song has been dissected into many different parameters (Table I). Different terms have been used to describe the same parameter by different authors. To simplify the discussion, here I will use one term for each parameter, and in several cases, that term is different from that used by the authors of a study.

The relevance of particular song parameters to mate recognition differs among species. For the *melanogaster* group, the most important parameter is the interpulse interval (IPI), the amount of time between each pulse (Ewing and Bennet-Clark, 1968). Mean IPI is species-specific and around 35 ms in *D. melanogaster* (Cowling and Burnet, 1981; Kawanishi and Watanabe, 1980). IPI cycles in a sinusoidal fashion, the K&H cycle, increasing and decreasing over approximately a minute in *D. melanogaster* song (Alt *et al.*, 1998; Kyriacou and Hall, 1980). The K&H cycle also influences mate choice such that a song without a K&H cycle is not preferred to no song (Ritchie *et al.*, 1999). Both *D. melanogaster* and *D. simulans* females greatly prefer their own IPI matched with their own K&H cycle (Kyriacou and Hall, 1982).

Table I. Courtship Song Traits

Abbreviation	Trait
AMP-RT	Amplitude ratio of sine to pulse song
CL	Cycle length
CPP	Cycles per pulse
FFT	Fast fourier transform, a profile of the pulse including CPP, IPF and additional frequencies
IPF	Intrapulse frequency or carrier frequency, equivalent to CPP/PL
IPI	Interpulse interval (time between the start of pulses), equivalent to PL + PAUSE
K&H cycle	The period of the IPI cycle
PA	Pulse amplitude
PAUSE	Time between pulses
PL	Pulse duration or pulse length
PN	Pulse number (number of pulses per train)
PTL	Pulse train length
SDUR	Duration of a sine song burst
SSF	Sine song frequency

Pulse structure differs among *Drosophila* species and can influence mating behavior. Although species of the *melanogaster* group do not differ greatly in pulse characters, the major characters differing between *D. virilis* and *D. lummei* include number of pulses in a pulse train (PN), the length of a pulse train (PTL), IPI, cycles per pulse (CPP), the length of a sound pulse (PL), the carrier frequency of the pulse, or intrapulse frequency (IPF) as well as IPI (Hoikkala and Lumme, 1987). *D. montana* females, which are closely related to *D. virilis* and *D. lummei*, choose their mates on the basis of pulse characters such as PL, CPP and IPF (Aspi and Hoikkala, 1995; Ritchie *et al.*, 1998).

Fast Fourier transform (FFT) has been used to analyze pulses in great detail (e.g. Wheeler *et al.*, 1988, 1989). FFT analysis produces a profile of the pulse including CPP, IPF and the additional frequencies in a pulse. The pulse shapes produced from a FFT analysis have been studied with several different mutations (see below).

SINGLE GENES AFFECTING COURTSHIP SONG

Many processes may influence song production including sex determination, neuroanatomy, wing musculature, timing of wing vibrations and hearing. None of the genes found to influence song production (Table II) only affect song, so the likelihood that there are "song genes" is slim. Even so, natural allelic variation may exist in genes influencing song that do not disrupt other functions. Through transformation of *D. melanogaster* with the gene of other species, allelic change has been demonstrated for two genes thus far (see below). Overall, general patterns in the types of genes influencing song may provide clues as to what kinds of genes should be tested for song defects in the future.

Genes Involved in Regulation

The *period* (*per*) locus is perhaps the best-described gene affecting courtship song. Discovered for its effects on circadian rhythm (Konopka and Benzer, 1971), *per* was subsequently demonstrated to affect the K&H cycle (Kyriacou and Hall, 1980). Mutations that make the circadian rhythm shorter, longer, or more arrhythmic than normal have the same effect on the K&H cycle (Alt *et al.*, 1998; Kyriacou and Hall, 1980, 1986, 1989).

Table II. Genes with Effects on Courtship Song in *Drosophila melanogaster*

Locus	Traits affected ^a	Molecular characterization ^b	Chromosome	Reference ^c
<i>period (per)</i>	K& H cycle	Transcription corepressor	X	1
<i>cacophony (cac)</i>	CPP, FFT, IPI, PA, sine song, PA, FFT	Voltage-gated calcium channel	X	2–5
<i>no-on transient A (nonA)</i>	CPP, FFT	RNA binding protein	X	3,6
<i>paralytic (para)</i>	IPI	Voltage-gated sodium channel	X	4
<i>ariadne (ari-1)</i>	no song	Ubiquitin protein ligase	X	7
<i>touch-insensitive-larva-B (tilB)</i>	AMP-RT, SSF	Unknown	X	8
<i>beethoven (btv)</i>	AMP-RT, CPP, PD, SSF	ATPase activity	2L	8
<i>maleless (mle)</i>	IPI, enhances <i>cac</i> IPI defects	Double stranded RNA binding domain	2R	5
<i>croaker</i>	CPP, IPI	Unknown	2R	9
<i>temperature-induced-paralytic-E (tipE)</i>	IPF, PA	Voltage-gated sodium channel	3L	5
<i>Cysteine string protein (Csp)</i>	CPP, IPF, PA	ATPase activity	3R	5
<i>transformer (tra)</i>	CPP, FFT	Pre-mRNA splicing factor	3R	4
<i>doublesex (dsx)</i>	IPI, sine song	Transcription factor	3R	10
<i>atonal (ato)</i>	AMP-RT, CPP, PL, PN	Transcription factor	3R	8
<i>fruitless (fru)</i>	FFT, IPI, song production	RNA polymerase II transcription factor	3R	3, 4, 11
<i>ebony (e)</i>	IPF, sine song	Beta-alanyl-dopamine synthase	3R	12
<i>slowpoke (slo)</i>	CPP, IPF, IPI, PA	Calcium activated potassium channel	3R	5

^aThe song trait affected by alleles at the locus are listed. See Table I for abbreviations. “Sine song” and “song production” indicates that the amount of song produced (sine song, or sine song and pulse song, respectively) is altered, always with the mutant alleles causing a reduction in singing.

^bMajor molecular characterization as given in Flybase (<http://www.flybase.org>).

^cMajor references for the effect on song: 1. Kyriacou and Hall, (1980; 1986; 1989); 2. von Schilcher (1976a; 1977); Kulkarni and Hall, (1987); 3. Wheeler *et al.*, (1989); 4. Bernstein *et al.*, (1992); 5. Peixoto and Hall (1998); 6. Kulkarni *et al.*, (1988); Rendahl *et al.*, (1992); 7. Aguilera *et al.*, (2000); 8. Tauber and Eberl, (2001); 9. Yokokura *et al.*, (1995); 10. Vilella and Hall, (1996); Taylor *et al.*, (1994); 11. Anand *et al.*, (2001); Ryner *et al.*, (1996); Vilella *et al.*, (1997); 12. Kyriacou *et al.*, (1978).

The *per* locus conveys species-specific behavioral instructions. Transformation of arrhythmic *per* mutants with the *D. simulans* gene results in normally cycling IPI song but with a *D. simulans* cycle length (Wheeler *et al.*, 1991). Moreover, the difference in cycling has been isolated to a specific region of the gene (Sawyer *et al.*, 1987; Wheeler *et al.*, 1991; Yu *et al.*, 1997). The *per* gene itself may not influence mate choice via the song, indicating that *per* has a minimal contribution to sexual isolation between the species (Ritchie and Kyriacou, 1994).

One of the few genes isolated through a screen for courtship song mutations is the *dissonance (diss)* allele of *no-on-transient-A (nonA)*, Kulkarni *et al.*, (1988). The *nonA* gene encodes an RNA binding protein (Jones and Rubin, 1990) and therefore regulates other genes. Many alleles of *nonA* are visual mutants with pleiotropic song effects (Rendahl *et al.*, 1992). The pulse train of a *nonA^{diss}* male starts normally, but pulses become more polycyclic over the length of the train (Kulkarni *et al.*, 1988). The pulse song structure of a *nonA^{diss}* male resembles the structure of *D. virilis* song in that the pulses are

polycyclic and the amplitude of the initial pulses in a train is higher than the amplitude of the concluding pulses (Hoikkala and Lumme, 1984). Transformation of *nonA⁻ D. melanogaster* with the *nonA* gene of *D. virilis* results in a fly that has a pulse structure similar to *D. virilis*, as well as a reduced IPI (Campesan *et al.*, 2001a, b). Because the visual abnormalities associated with *nonA⁻* are rescued by *D. virilis nonA*, the species-specificity of the behavior is not the result of incomplete rescue (Campesan *et al.*, 2001b).

Ion Channels

Ion channel genes are potential candidate genes for song because they play a role in membrane excitability. One such gene, the *cacophony (cac)* locus, encodes an $\alpha 1$ calcium-channel subunit with different isoforms that are generated by alternative splicing (Smith *et al.*, 1996, 1998). A sex-linked mutation, *cac* was the first song gene identified through a screen for courtship mutations (von Schilcher, 1976a). Although it affects other traits, the most notable effect of *cac* is on CPP (Kulkarni and Hall, 1987;

von Schilcher, 1976a, 1977; Wheeler *et al.*, 1989). The *cac* gene is potentially involved in song variation in *D. virilis* (see Quantitative Genetics, below). Interspecific transformation of the *cac* locus is difficult because it encodes a very large gene spanning over 45 kb with multiple splicing variants (Peixoto *et al.*, 1997). Transformation might be possible with a cDNA, but the appropriate transcript to use is not clear.

One other notable phenotype of *cac* pertains to the way the flies hold their wings. *D. melanogaster* males normally hold a single wing out at about 90° when singing. *cac* males hold their wings in an “opened, raised and twisted position” when held in a vial with females (von Schilcher, 1976a), a position used by other species (Spieth, 1952). As yet the effect of wing position on the production of species-specific song has not been investigated.

Other ion channels affect song including two voltage-gated sodium channels, *paralytic* (*para*) and *temperature-induced-paralytic-E* (*tipE*), and a calcium-activated potassium channel, *slowpoke* (*slo*; Peixoto and Hall, 1998). Although not an ion channel itself, *mle* affects sodium channel function and the *mle^{napts1}* allele both affects IPI and enhances the *cac* defects of IPI (Peixoto and Hall, 1998). Similarly, *Csp*, which is involved in neurotransmitter release and may interact with calcium channels (Mastrogiacomo *et al.*, 1994; Zinsmaier *et al.*, 1994), affects CPP, IPF and PA. However, not every ion channel has an effect on courtship song: a temperature-sensitive allele of *seizure* (*sei*), a potassium channel, lacks effects on courtship song (Peixoto and Hall, 1998).

Sex Determination Pathway Genes

A cascade of genes regulates sex determination in *Drosophila*. Just as these genes determine the morphological sexual identity of the fly, they also affect behavioral sexual identity (see Billeter *et al.*, 2002 for review). The first gene in the pathway, *Sexlethal*, controls the production of the *transformer* (*tra*) protein product. The *tra* mutant turns XX females into pseudomales and both XX *tra/tra* females and XY *tra/tra* males sing with the same abnormalities in their songs: CPP and FFT are altered (Bernstein *et al.*, 1992). The *tra* gene in turn regulates *doublesex* (*dsx*), which produces male and female-specific transcripts (see Billeter *et al.*, 2002 for review). The effect of *dsx* are subtle: *dsx* flies do not sing sine song and IPI is altered song (Taylor *et al.*, 1994; Vilella and Hall, 1996).

The TRA protein also regulates the *fruitless* (*fru*) gene (see Billeter *et al.*, 2002 for review). Many alleles of *fru* alter the courtship behavior of the males: males court females for less time than normal, court males, and can be defective in courtship patterns including courtship song (Anand *et al.*, 2001; Gailey and Hall, 1989; Goodwin *et al.*, 2002; Hall, 1978; Ryner *et al.*, 1996; Vilella *et al.*, 1997). Males carrying some alleles of *fru* extend their wings but do not sing whereas other alleles alter IPI (Anand *et al.*, 2001; Ryner *et al.*, 1996; Vilella *et al.*, 1997; Wheeler *et al.*, 1989). Because *fru* specifies sexual differentiation in the central nervous system responsible for male sexual behavior, it has been proposed to be a clear “behavior gene” (Baker *et al.*, 2001). To determine what role this gene might play in species differentiation through interspecific gene transformation, such as with *per* and *nonA*, is difficult because the gene is large with multiple transcripts (Kyriacou, 2002; Ryner *et al.*, 1996) although the use of cDNA might be possible.

Auditory Mutations

Chordotonal organs present in the antenna and wings (Miller, 1950) allow males to hear their own song or perceive a proprioceptor response from the wing (Tauber and Eberl, 2001). Three auditory mutations, *atonal* (*ato*), *beethoven* (*btv*) and *touch-insensitive-larvae-B* (*tilB*), disrupt the chordotonal organs and also affect courtship song (Tauber and Eberl, 2001). Because these genes affect both production and perception of song, these genes may play a role in sender-receiver co-evolution (Tauber and Eberl, 2001), although the genes have not been investigated in any other *Drosophila* species.

These auditory genes also affect the way the songs are produced. Many *ato* and *btv* flies vibrate two wings at a time (wildtype vibrate only one) and *btv* flies often twist their wings to a 90° angle (Tauber and Eberl, 2001) whereas wildtype *D. melanogaster* twist the wing so that the trailing edge is slightly lower than the leading edge but otherwise parallel to the body (Ewing, 1977). Such types of wing movements have been observed in other species of *Drosophila* (Spieth, 1952) and thus may be good candidate genes for studying species differences in courtship.

Other auditory mutants include *aristaless* (*al*) and *thread* (*th*). Double mutants for *aristaless* (*al*) and *thread* (*th*) lack functional arista and cannot perceive song (Burnet *et al.*, 1971, 1977). The phrases (a unit of sine song with a pulse song train) are shorter in

al;th flies than in both wildtype flies and flies that have had their arista surgically removed (Burnet *et al.*, 1977). Because flies that have their arista surgically removed sing normally, this implies that acoustic feedback through the arista is not needed and therefore, *al* and *th* potentially directly influencing song production (Burnet *et al.*, 1977). With the more advanced analyses of song since the 1970s (e.g. FFT analysis), the *al* and *th* mutations should be re-examined to determine if they affect aspects of pulse structure.

Genes Affecting Flight

Because song production involves the same direct and indirect muscles as those required for flight (Ewing, 1977, 1979), mutations that affect flying ability sometimes also affect song production. For example, flies carrying the *croaker* (*cro*) mutation have trouble flying, indicating that it is a general defect in the musculature (Yokokura *et al.*, 1995). One of the few genes identified in a screen for mutations affecting courtship song, *cro* alters CPP and IPI (Yokokura *et al.*, 1995). *cro* has not yet been molecularly characterized.

Another mutation affecting flight is *ariadne* (*ari-1*), a gene important in neural development (Aguilera *et al.*, 2000). Flies carrying mutations in *ari-1* have a lower than normal wing beat frequency and cannot fly. Males do not sing, but neither do they perform any of the normal courtship routines (Aguilera *et al.*, 2000). Because of overall locomotor defects in *ari-1* flies, this is probably not an important gene in song production.

Other genes that affect flight do not affect song production. Two mutations, *ISO-Q* and *KA16-II*, affect wing-beat frequency but have songs within the normal range for SSF, IPF, CPP and IPI (Barnes *et al.*, 1998). Similarly, *cac* does not affect flight (Kulkarni and Hall, 1987). Thus, defects in song and flight do not always have pleiotropic effects on each other.

Body Color Mutation

The *ebony* (*e*) gene is a beta-alanyl-dopamine synthetase that affects the color of the body (Wright, 1987). The gene affects IPF and the amount of sine song produced (Kyriacou *et al.*, 1978). There are also other effects on IPI and SSF, but these are dependent upon the age of the fly and the genetic background (Kyriacou *et al.*, 1978). More recently an effect of *e*

on circadian period has been found (Newby and Jackson, 1991). The period of the IPI cycle has not been tested in *e* flies, though given the circadian effect, there might be an effect of *ebony* and it should be examined.

QUANTITATIVE GENETICS

Many studies of courtship song have taken a quantitative genetics approach (Table III). In these cases, the intent is to analyze the natural standing variation in a population of a species or to examine the genetics of species differences for the behavioral trait. The courtship song trait studied varies by species group as most studies focus on the most important courtship song trait for mate recognition in that group. The quantitative genetics approach can test for the possible contribution of candidate genes identified through the studies above. Co-occurrence of factors and candidate genes is not sufficient to show that a particular gene is involved in a trait, but does indicate that a particular gene warrants further investigation.

Quantitative genetics also enables study of species that are genetically intractable for single gene analysis. Comparison of results among species groups is difficult, partly because the most interesting song characters differ among species groups. Studies have been performed in the *melanogaster* group, including the *melanogaster* subgroup and the *auraria* complex, in the *obscura* group (*D. pseudoobscura* and *D. persimilis*), the *repleta* group (*D. mercatorum*) and among various members of the *D. virilis* group.

D. melanogaster Group

Most studies of *D. melanogaster* find additive autosomal effects for IPI (Colegrave *et al.*, 1999; Cowling, 1980; Gleason *et al.*, 2002) with the exception of a study of *D. melanogaster* using lines selected for long and short mean IPI in which the X and autosomes had significant effects (Ritchie and Kyriacou, 1996). Of the autosomes, both Colegrave *et al.*, (1999) and Gleason *et al.*, (2002) found larger effects on the 3rd chromosome and smaller effects on the 2nd chromosome. In the latter study, using quantitative trait loci (QTL) analysis, only one of the candidate genes (Table II), *tipE*, is located in the same area as a QTL. Interestingly, *tipE* has not been demonstrated to affect IPI. The lack of concordance between QTL and candidate genes implies that the analysis of genes that alter song when mutated has

Table III. Quantitative Genetics Studies of Courtship Song

Species/method of study	Trait ^a	Findings	Candidate ^b
<i>D. melanogaster</i>			
Diallel analysis of 4 strains ¹	SSF, IPI	Additive effects	
Selection and REML ^{c,2}	IPI	Additive genes throughout genome	
Cosmopolitan and African chromosome substitution lines ³	IPI	Large effect of 3rd chromosome with other factors on the 2nd chromosome, epistasis	
QTL mapping with RI lines ⁴	IPI	One QTL on 2L, 2 QTL on 3L	<i>tipE</i>
<i>D. melanogaster</i> – <i>D. simulans</i>			
Hybrids ⁵	IPI	Autosomal genes, no dominance	
Hybrids ⁶	IPI	Autosomal	
	K&H cycle	X linked	<i>per</i>
	Amplitude of K&H cycle	Autosomal	
<i>D. simulans</i> – <i>D. mauritiana</i>			
Backcrosses, one marker per chromosome arm ⁷	IPI	Additive genes throughout genome	
<i>D. simulans</i> – <i>D. sechellia</i>			
QTL mapping of backcross hybrids ⁸	IPI	4 QTL on 2R, 2 QTL on 3R, minor effect genes	<i>mle, cro, fru</i>
<i>Various D. melanogaster group species</i>			
Hybrids ⁹	SSF	X chromosome	
	IPF	Autosomal	
	IPI	Autosomal, possible dominance effects	
<i>D. virilis</i>			
Joint scaling test and contrast analysis of variance : hybrids, Backcross hybrids and reciprocal F ₂ hybrids ¹⁰	PN	Additive, dominance, additive x additive maternal interaction, on X and autosomes	
QTL mapping of F ₂ individuals of Different strains ¹¹	PTL	Autosomes only, Y chromosome effects	
	PN	1QT: on X, 1 QTL on 2, 5 QTL on 3	
	PTL	4 QTL on 3	
<i>D. montana</i>			
Inbred and outbred strain hybrids ¹²	CPP, IPF, IPI, PL, PN, PTL	Autosomal effects CPP, PL: dominance, no epistasis IPF: dominance and dominance x dominance epistasis	
Diallel analysis ¹³	PN, PTL, IPI, PL, CPP, IPF	Significant additive variance in PN, IPI, CPP, IPF, dominance in CN and IPF, no significant variation in PL	
<i>D. littoralis</i>			
Strain hybrids and backcross hybrids with limited markers ¹⁴	CPP, IPF	Large contribution of chromosome 2 with non-additive interactions between chromosomes 2 and 3–4 IPF: small effect of chromosome 5	
	IPI	X chromosome effect	
<i>D. virilis</i> – <i>D. lummei</i>			
Hybrids and backcross hybrids with visible markers ¹⁵	PN	Additive genes on all autosomes, no effect of X chromosome	
<i>D. virilis</i> – <i>D. littoralis</i>			
Hybrids and backcross hybrids ¹⁶	CPP, IPF, PL, PN, PAUSE, PTL	Effect of the proximal third of the X Chromosome for all traits, fused 3–4 chromosome has an effect on PAUSE	
Backcross hybrids ¹⁷	CPP, IPF, PL, PAUSE, PN, PTL	Proximal part of X for CPP, PL, PAUSE, PN	<i>nonA, cac</i>

Table III (Continued)

Species/method of study	Trait ^a	Findings	Candidate ^b
<i>virilis</i> phylad (Five species) Diallel analysis ¹⁸	CL, CPP, IPI, PN, PTL	Largely autosomally determined, significant dominance in all traits except PL; X or cytoplasmic effects for PTL, IPI, PL, CPP	
<i>D. virilis</i> - <i>D. montana</i> ,- <i>D. littoralis</i> , <i>D. flavomontana</i> Hybrids ¹⁹	PL PAUSE	X chromosome major effect Large X effect with epistatic interactions	
<i>D. flavomontana</i> ,- <i>D. montana</i> , <i>D. littoralis</i> ,- <i>D. virilis</i> Hybrids, F ₂ ²⁰	PL PAUSE	Epistatic interactions between X and autosomes Large X effect	
<i>D. auraria</i> complex Hybrids ²⁰	IPI	X/Y and/or cytoplasmic effects and autosomal effects	
<i>D. auraria</i> - <i>D. bauraria</i> Backcross hybrids with 3 markers ²⁰	IPI	Additive autosomal effects	
<i>D. pseudoobscura</i> - <i>D. persimilis</i> Hybrids and backcross hybrids ²¹ Hybrids and backcross hybrids ²²	Song type IPI IPF	X chromosome effects X chromosome and autosomal effect X chromosome and autosomal effect	
QTL mapping of backcross hybrids ²³	IPI IPF	2 QTL on X chromosome, 1 QTL on 2nd chromosome 1 QTL on X chromosome, 1 QTL on 2nd chromosome	
<i>D. mercatorum</i> Hybrids ²⁴	A song IPI B song IPI	Autosomal Large X effect with autosomal effects	

^aAbbreviations are given in Table I.

^bCandidate genes are listed in Table II.

^cREML: residual maximum likelihood.

References: 1. Cowling, (1980); 2. Ritchie and Kyriacou, (1996); 3. Colegrave *et al.*, (1999); 4. Gleason *et al.*, (2002); 5. Kawanishi and Watanabe, (1981); 6. Kyriacou and Hall, (1986); 7. Pugh and Ritchie, (1996); 8. Gleason and Ritchie, (2004); 9. Cowling and Burnet, (1981); 10. Huttunen and Aspi, (2003); 11. Huttunen *et al.*, (2004); 12. Aspi, (2000); 13. Suvanto *et al.*, (2000); 14. Hoikkala and Lumme, (1990); 15. Hoikkala and Lumme, (1984); 16. Hoikkala *et al.*, (2000); 17. Päällysaho *et al.*, (2001); 18. Hoikkala and Lumme, (1987); 19. Päällysaho *et al.*, (2003); 20. Tomaru and Oguma, (1994a); 21. Ewing, (1969); 22. Noor and Aquadro, (1998); 23. Williams *et al.*, (2001); 24. Ikeda *et al.*, (1980).

not yielded much information about the natural variation underlying courtship song, at least in *D. melanogaster*. However, this is only one QTL study of two strains and thus may not represent the range of variation that exists in nature. More studies of natural variation within *D. melanogaster* are needed.

A clear pattern to IPI variation in *D. melanogaster* is apparent in that all of the mutations that change IPI, lengthen IPI. A similar pattern is evident from the quantitative genetics studies. In a selection experiment, there was a larger response to selection for increased IPI than for decreased IPI (Ritchie and Kyriacou, 1996). Among the recombinant inbred lines used for

QTL mapping, there were more lines with longer IPI rather than shorter IPI in comparison to the original parental lines (Gleason *et al.*, 2002). This pattern has been taken to be indicative to sexual selection for short IPI (Gleason *et al.*, 2002; Ritchie and Kyriacou, 1996), but female intraspecific preferences have not been tested. African *D. melanogaster* lines have a much shorter mean IPI than cosmopolitan lines (Colegrave *et al.*, 1999), well beyond the limit of selection (Ritchie and Kyriacou, 1996), implying that there are different alleles present for IPI in the African lines and these lines should be studied in more detail.

Hybrids and backcrosses within the *D. melanogaster* group have shown the same pattern

of autosomal contribution to IPI (Cowling, 1980; Cowling and Burnet, 1981; Gleason and Ritchie, 2004; Kawanishi and Watanabe, 1981, 1980; Kyriacou and Hall, 1986) with the exception of a study between *D. simulans* and *D. mauritiana* that could not exclude a contribution of the X chromosome (Pugh and Ritchie, 1996). However, the K&H cycle is X-linked (Kyriacou and Hall, 1986) and the X-linked factor is the *per* locus (Wheeler *et al.*, 1991).

A QTL study of the IPI difference between *D. simulans* and *D. sechellia* found different QTL than those within *D. melanogaster* for the same trait (Gleason and Ritchie, 2004). The lack of an overlap with QTL for *D. melanogaster* mean IPI implies that the genes contributing to intraspecific variation are different from the genes contributing to interspecific differences. The comparison is not completely valid: a proper comparison should be made to an intraspecific QTL study of *D. simulans*, but this has not yet been done. Regardless of the outcome, the results of a *D. simulans* QTL study will be informative. One possibility is that the QTL will match those that differ between *D. simulans* and *D. sechellia*, indicating that the same genes contribute to intraspecific variation and interspecific differentiation, yet intraspecific variation differs between species because *D. melanogaster* and *D. simulans* would not have the same QTL for mean IPI. An alternative is that *D. melanogaster* and *D. simulans* do have the same QTL for mean IPI and the same genes do not contribute to both intraspecific variation and interspecific differentiation. A third alternative is that new QTL would be found.

***D. virilis* Group**

The quantitative genetics of courtship song are perhaps understood much better in the *virilis* group than in another other *Drosophila* group. The group is particularly amenable to the study of courtship song because of the amount of variation in song and the ease of crosses between species of even different phylads. In this group, IPI is relatively constant and not important to mate recognition whereas characters of the pulse trains, such as PN and PTL, as well as pulse structure traits, such as CPP and IPF, are variable and important (Aspi and Hoikkala, 1995; Hoikkala and Lumme, 1984, 1987; Hoikkala *et al.*, 1998; Liimatainen *et al.*, 1992). Although IPI is not important to mate recognition, it does play a role species recognition (Saarikettu *et al.*, 2005).

In intraspecific studies of courtship song in the *virilis* group, most traits are influenced by factors on

the autosomes (Table III: Aspi, 2000; Hoikkala and Lumme, 1990; Huttunen and Aspi, 2003; Huttunen *et al.*, 2004; Suvanto *et al.*, 2000). An exception is an X effect on PN in *D. virilis* (Huttunen *et al.*, 2004). Despite little effect of the X in intraspecific crosses, the X has significant effects on CPP, IPF, PL, PAUSE (time between pulses, not including the pulse) and PN in crosses between *D. virilis* and *D. littoralis* (Hoikkala *et al.*, 2000). This X effect is localized to the proximal end of the X chromosome and is associated with the song candidate genes *nonA* and *cac* (Päällysaho *et al.*, 2001). Thus, as in the *melanogaster* group the loci contributing to variation in song within a species (*D. virilis*) are not necessarily the same as those contributing to species differences (*D. virilis*-*D. littoralis*). Whether or not this is a general pattern in the *virilis* group remains to be determined. The difference in PN between *D. virilis* and *D. lummei* has a polygenic autosomal inheritance with no X chromosome effect (Hoikkala and Lumme, 1984), whereas there is an X effect for *D. virilis* (Huttunen *et al.* 2004). Planned QTL experiments will determine whether the intraspecific and interspecific difference holds (Huttunen *et al.*, 2004).

Crosses among the species of the *virilis* and *montana* phylads (Hoikkala and Lumme, 1987) are revealing autosomal mode of inheritance for most traits although a contribution of the X depends upon the cross performed: crosses between the phylads tend to have an X component influencing some traits. Comparing various hybrids with *D. virilis* and *D. flavomontana*, the contribution of the X is different for these two species: *D. flavomontana* had a strong X effect on PAUSE where as *D. virilis* does not (Päällysaho *et al.*, 2003). For PL, *D. flavomontana* autosomes interacted with the X chromosome, whereas there was a stronger X component with *D. virilis*. Thus, even within species groups, there are different modes of inheritance of song characters with different genes contributing to the same traits.

Other Species

There are many other excellent studies of the quantitative genetics of courtship song in other species, but for a lack of space, cannot be covered in detail here (see Table III for results). A few patterns are of note. The first is the lack of X effects on IPI for the *melanogaster* group. The *auraria* complex is similar to other *melanogaster* group species in that the predominant effects on IPI for *D. auraria* are associated with the autosomes, although crosses between

some species pairs have significant X/Y and/or cytoplasmic effects (Tomaru and Oguma, 1994b).

A second pattern is the association of song QTL with inversions. QTL mapping of song differences between *D. pseudoobscura* and *D. persimilis*, indicates that the QTL are most strongly associated with inversions fixed between the two species (Williams *et al.*, 2001). Hybrid male sterility and female species preferences are also associated with inversions in these species, thus linkage groups maintained by inversions may hold the species differences (Noor *et al.*, 2001). Some of the courtship song differences between *D. virilis* and *D. littoralis* are also associated with inversions (Hoikkala *et al.*, 2000).

A third pattern that is emerging is that different types of songs within a species may be affected by different genes. Many *Drosophila* species outside of the *melanogaster* group have two types of pulse song (e.g. Ewing, 1969; Ewing and Miyan, 1986; Ritchie and Gleason, 1995). In *D. mercatorum*, the two pulse song types are controlled by different sets of factors located in different parts of the genome (Ikeda *et al.*, 1980). More studies are needed on species with two pulse song types to see if independent control of the song types is a general pattern, including more studies of sine song within *D. melanogaster*.

PROSPECTS

The traditional genetic mutation analyses have provided good candidate genes for within and between species variation, however, in general the genes do not overlap with QTL. More resolution is needed in the quantitative genetics studies to determine what genes are contributing to courtship song variation. The quantitative genetics studies are showing that different genes are probably contributing more to important species differences. For example, the X chromosome is not important for IPI variation in *D. melanogaster*, but the X chromosome is important for pulse structure characters in *D. virilis*.

More courtship song genes are needed, but screening for mutations affecting courtship song is laborious. Only three of the known courtship song mutations, *cac*, *nonA^{diss}* and *cro*, were isolated through genetic screens. For *cac* and *cro*, the mutations were screened for males that were not readily accepted by females (von Schilcher, 1977; Yokokura *et al.*, 1995). Males defective in courtship were subsequently analyzed for song. Thus, these mutations must have had a large effect to be identified. Only in the case of *nonA^{diss}* were males (2500 lines) directly

analyzed for song (Kulkarni *et al.*, 1988). Because courtship song is such a complex phenotype to score, it is not possible to screen the thousands of mutations necessary for a standard screen; thus, other approaches are needed to identify more candidate genes.

The *Drosophila melanogaster* genome project, although providing a wealth of information for marker development useful in QTL analysis, has not yielded any insights into the genetics of courtship song. For traits such as cuticular hydrocarbons profile, which affects mating discrimination (e.g. Ferveur, 2005), the genome sequence provides clues of possible candidate genes through similarity to known enzymes involved in hydrocarbon biosynthesis (Gleason *et al.*, 2005). Not enough is known about genes in behavioral pathways to guess from the sequences which genes might be potential song genes. The ion channel genes indicate a potential role for that particular class of gene, however a search of Flybase (<http://www.flybase.org>) for calcium channel genes yields a total of 61 genes, of which 18 are novel sequences from the sequencing project. To test the genes, mutations are needed. The Berkeley *Drosophila* Gene Disruption Project (Bellen *et al.*, 2004) is creating P-element mutants in as many genes as possible, and this may provide the resources for finding more courtship song mutations. This approach would be most fruitful with more information about the pathways of the known genes for generating the phenotypes observed so that appropriate gene types might be tested.

Gene disruption might not be the best approach. Many mutations that cause major disruptions result in phenotypes that are more severe than those found in nature. Many of the alleles that affect *Drosophila* behavior are mild effects of genes with severe phenotypes (Greenspan, 1997). The subtle effects of many of the genes implicated in courtship song would not have been found by only studying null alleles. For example, if only the *per⁰* mutation had been found, the lengthening and shortening of the K&H cycle would not have been found, although the elimination of the cycle would have been detected. Thus, it is possible that some of the genes that completely eliminate song might have alleles with small effects on other song characters.

Alterations in protein activity and levels of expression may have profound effects on behavior without detectable changes on other phenotypes. Such changes resemble behavioral variants and thus are also more relevant to species-specific behavioral

changes. Screening for subtle variants in courtship song is not technically feasible because of the small changes that natural variants effect. For example, the estimated effects of the IPI QTL for *D. melanogaster* song are less than a millisecond (Gleason *et al.*, 2002). Because of the amount of environmental variance in courtship song, measuring these effects requires very large sample sizes.

Changes in gene expression may be one approach to identifying courtship song genes. Microarray analysis has been used to identify genes affecting geotaxis in *D. melanogaster* (Toma *et al.*, 2002). In this case, lines selected for opposite geotaxis were compared. The same should be possible with *D. melanogaster* lines selected for courtship song traits. Unfortunately, the IPI selection lines have been lost and will need to be reselected (M.G. Ritchie, pers. comm.).

As has been suggested (Kyriacou, 2002), transformation of *D. melanogaster* with genes that are associated with different song structures in other species may potentially yield insights into the natural variants of song. However, good candidate genes are few and many are impractical for transformation because of large or complex molecular structure (such as *cac* and *fru*). However, continued QTL work in the *virilis* group has great potential for identifying good candidate genes because of the large effects between species. The completion of the *D. virilis* genome will greatly aid in candidate gene identification in *virilis* group species.

Another promising approach to single gene analysis uses information from mosaics to determine the regions of the fly involved in particular behaviors. Through sex mosaics, pulse song was found to be controlled in the mesothoracic ganglion (von Schilcher and Hall, 1979). Interestingly, sine song is associated with both the brain and thoracic ganglion, but the exact location has not been determined (von Schilcher and Hall, 1979). The difference between the song types correlates with the involvement of different muscles for each song type (Ewing, 1979) and the effects of genes on only one or the other song type (Table II). Other expression mosaics have identified regions of the mushroom body with effects on courtship song production (thought characteristics of song itself were not measured, Broughton *et al.*, 2004). As genes are further characterized for their expression patterns in particular regions of the fly, additional candidate genes may become evident.

With the additional *Drosophila* genomes that are being sequenced, other *Drosophila* species are

becoming viable candidates for the study of the genetics of courtship song. One particularly promising species is *Drosophila paulistorum*, a member of the *willistoni* group. The *D. willistoni* genome should be complete enough for the relatively easy development of markers within a year. *D. paulistorum* has been a great model species for the study of reproductive isolation (e.g. Ehrman, 1961, 1965; Kim *et al.*, 1992). The species has six semispecies, some of which can form fertile hybrids and some of which do not (reviewed in Ehrman and Powell, 1982). The *D. paulistorum* semispecies have simple pulse songs, each with a unique IPI (Ritchie and Gleason, 1995). Work is currently underway to analyze the quantitative genetics of semispecies hybrid songs (Gleason, unpublished). The same approach will also be possible in many other species with other types of songs and for which different characters are important for song recognition. Ultimately, the combined approach of single gene analysis, quantitative genetic analysis and the use of different species should provide insights into this complex and biologically important trait.

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