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REVIEW

Behavioral genomics and the study of speciation at a porous species boundary**

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Summary

Porous species boundaries are characterized by differential gene flow, where some regions of the genome experience divergent evolution while others experience the homogenizing effects of gene flow. If species can arise or remain distinct despite gene flow between them, speciation can only be understood on a gene by gene level. To understand the genetics of speciation, we therefore must identify the targets of selection that cause divergent evolution and identify the genetic architecture underlying such "speciation phenotypes". This will enable characterization of genomic regions that are "free to flow" between species, and those that diverge in the face of gene flow. We discuss this problem in the genus *Laupala*, a morphologically cryptic, flightless group of crickets that has radiated in Hawaii. Because songs are used in courtship and always distinguish close relatives of *Laupala* as well as species in sympatry, we argue that songs in *Laupala* are speciation phenotypes. Here, we present our approaches to identify the underlying genomic regions and song genes that differentiate closely related species. We discuss what is known about the genetic basis of this species difference derived from classic quantitative genetics and quantitative trait locus mapping experiments. We also present a model of the molecular expression of cricket song to assist in our goal to identify the genes involved in song variation. As most species are sympatric and exchange genes with congeners, we discuss the importance of understanding the genetic and genomic architecture of song as a speciation phenotype that must be characterized to identify differential patterns of gene flow at porous species boundaries.

Key words: species boundaries, speciation, *Laupala*, speciation genes, phenotype, hybridization, gene flow, genomics, behavior, crickets

Introduction

Founders of the modern evolutionary synthesis devoted enormous research effort to developing a model of species as biological units and concluded that the adaptive boundaries of species were sealed to gene flow from other such species (Dobzhansky, 1937; Mayr, 1942). However, our perspective on the species boundary, i.e., the species' limits of interbreeding and gene flow, has changed in recent years. Empirical insights gained with molecular tools suggest that some degree of gene flow between closely related species is widespread (e.g., Grant and Grant, 1998; Schluter, 1998; Mallet et al., 1998; Rieseberg et al. 1999; Shaw, 2002; Mendelson and Shaw, 2002; Sota et al., 2001). Furthermore, recent theoretical work suggests that divergence leading to speciation can occur in the face of gene flow (Doebeli and Dieckmann, 1999; Kondrashov and Kondrashov, 1999; Doebeli and Dieckmann, 2003). This is most strongly exemplified by theoretical models of "sympatric" speciation where divergence occurs even when spatial and temporal mixing is complete.

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However, directional selection on complex phenotypes in the face of gene flow is equally astounding when nascent species are partially separated geographically or seasonally (e.g., Schneider et al., 1999; Danley et al., 2000; Filchak et al., 2000; Danley and Kocher, 2000; Ogden and Thorpe, 2002). Under such conditions, gene flow continues at some level, carrying the potential to break up the coadapted gene complexes underlying the phenotypes upon which selection acts. That species can continue to diverge under these conditions is therefore surprising and has lead to the idea that boundaries are porous among recently diverged species. Porous species boundaries are characterized by differential gene flow, where some regions of the genome can experience divergent evolution while others experience the homogenizing effects of gene flow.

"Speciation phenotypes" are targets of selection that cause divergent evolution and speciation. Determining the genetic architecture of the speciation phenotypes under directional selection (including the number, location, directionality, and magnitude of effect of genes underlying divergent phenotypes) is crucial to identifying the differential, gene by gene, patterns of gene flow that characterize speciation. In newly evolved species, chromosomal regions that harbor genes underlying such phenotypes should show the effects of an early reduction in gene flow, while other, "neutral" regions continue to flow across species boundaries. Thus, in order to understand the genetics of speciation, the genes that underlie the divergent phenotypic evolution causing a reduction in gene flow must be identified and localized.

The most direct way to study the phenotypes of such early divergence processes is by a "forward" genetics approach, which seeks to identify genes underlying divergent phenotypes that cause a reduction in gene flow between nascent species. A forward genetics approach targets the divergent phenotypes the function of which is already known or hypothesized within species. This contrasts to the traditional "reverse" genetics approach that works back from relative, non-functional sterility or inviability phenotypes, to identify the functional phenotypes of these genes within species (reviewed in Orr and Presgraves, 2000). Sterility and inviability are not phenotypes that selection can favor (Coyne, 1974; Rieseberg et al., 1995); their study does not directly investigate the target of divergent selection that results in speciation.

In this paper, we describe our research efforts to obtain a genic understanding of speciation phenotypes through a forward approach, in a group that is likely characterized by porous species boundaries. We propose a model for the expression of this phenotype for use in identifying the underlying genes involved in differentiation and speciation.

The genetics of speciation in the Hawaiian cricket genus Laupala

The genus *Laupala* comprises 37 flightless species of crickets endemic to the Hawaiian archipelago, part of a larger radiation of trigonidiine crickets that has resulted in over 160 endemic species (Otte, 1994; Shaw, 2000). The distribution of each species of *Laupala* is limited to the mid-elevation rainforests of a single island, and often to a single volcano within that island. Morphologically, all species are cryptic and appear to have similar ecologies and reproductive behavior. The three species groups comprised by the genus are formally recognized on the basis of small metric differences in the male genitalia. Close relatives within species groups are distinguished by differences in the pulse rate of the male calling song (Fig. 1; Otte, 1994). DNA sequence variation has corroborated these species groups and suggests further that two of these groups radiated from older to younger islands in the archipelago (Shaw, 2002). Both inter- and intra-island speciation events have occurred, but current data suggest that speciation has occurred primarily (if not exclusively) in allopatry.

Like other crickets where the mate location and courtship function of the acoustic communication system has been studied (e.g. Alexander, 1962; Huber and Thorson, 1985; Stabel et al., 1989), *Laupala* males use song extensively in pair formation and courtship, and females are most attracted to pulse rates of their own species (Shaw, 2000; Shaw and Herlihy, 2000). In addition, closely related, allopatric sister species always differ in the pulse rate of the male calling song, sometimes quite substantially. This dramatic difference is exemplified by the close relatives *L. kohalensis* and *L. paranigra* (Fig. 1), both of which are endemic to the Big Island, the youngest Hawaiian island at ca. 400,000 years (Carson and Clague, 1995). This functional context in which song is displayed by males, in addition to the observation that song variants in *Laupala* have been evolutionarily fixed during, or soon after, lineage splitting suggests that songs in *Laupala* have diverged under selection. A potential selective mechanism for song evolution is interspecies interactions. The distributions of most species typically overlap with 1–3 congeners. Within these sympatric, congeneric communities, species always sing distinctively, varying most conspicuously in the pulse rate of the male calling song (Fig. 2).

Despite divergence in song between both closely related and sympatric species of *Laupala*, there is reason to believe that some degree of gene flow occurs between species. A comparison of species relationships based on nuclear and mitochondrial DNA suggests that mtDNA gene flow has occurred repeatedly between sympatric congeners. Seven independent locations where sympatric congeners have experienced interspe-

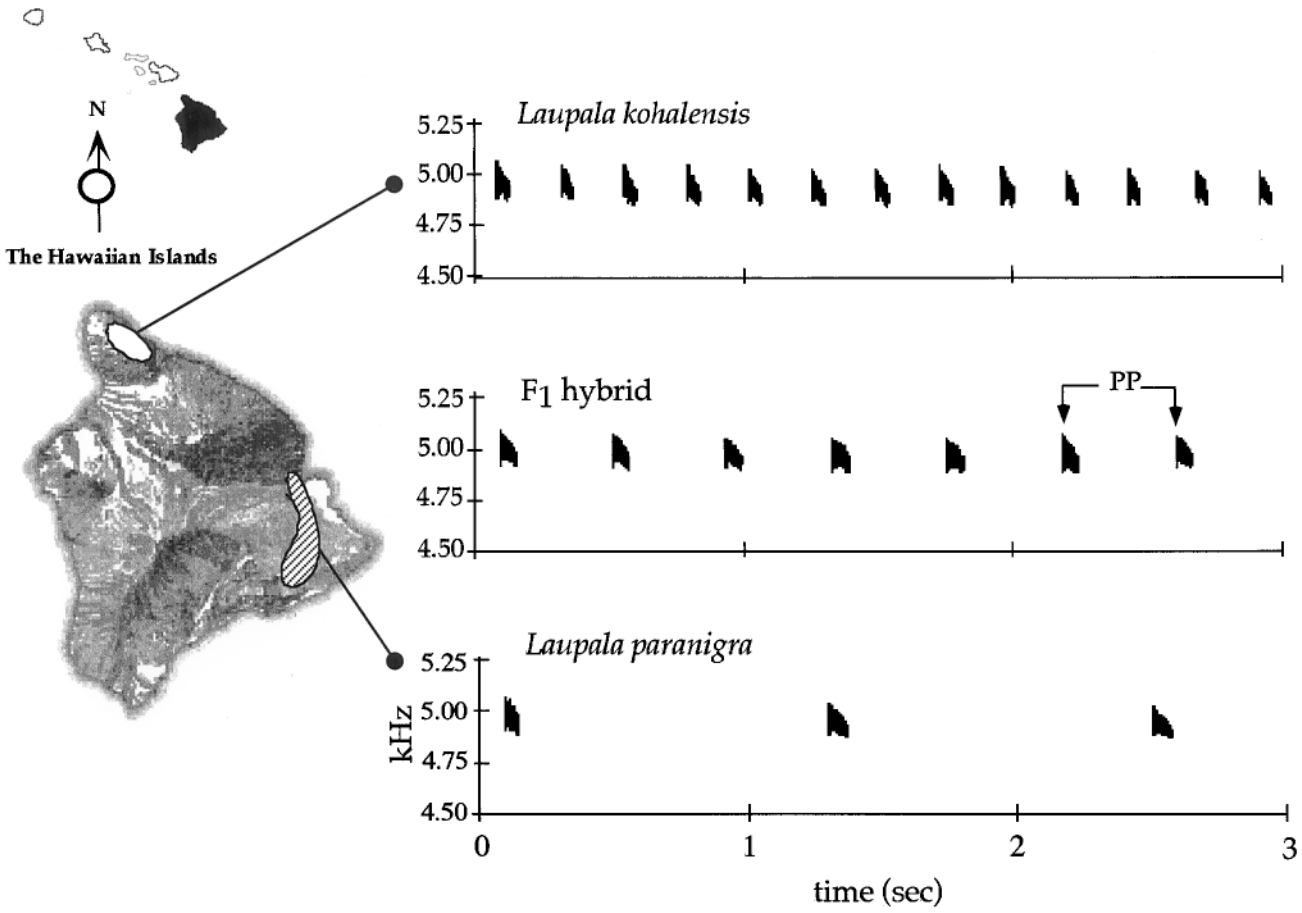

Fig. 1. Sonagrams of observed songs from *L. kohalensis* and *L. paranigra* and an F1 hybrid between them. Species distributions are indicated. All *Laupala* species possess trilling songs of this structure that primarily differ in pulse rate. PP = pulse period, the inverse of which = pulse rate.

cific mtDNA gene flow have been identified (Shaw, 2002). Two of these cases involve *L. cerasina*, at one location where it is sympatric with *L. paranigra* and at another with *L. kohalensis*. An examination of the species boundary between *L. kohalensis* and *L. cerasina* using Amplified Fragment Length Polymorphism (AFLP) variation shows numerous examples of shared polymorphisms between the two species in addition to some fixed differences (Mendelson and Shaw, 2002). The AFLP method generates genetic markers through the electrophoresis of amplified (via the polymerase chain reaction) restriction fragments. While the retention of ancestral polymorphism may explain the shared AFLP polymorphisms, shared variation due to recent nuclear gene flow seems plausible given that interspecific mtDNA gene flow has occurred between these species. Despite this, the male calling song and female acoustic preference remain distinct between these two species (Fig. 2; Mendelson and Shaw, 2002). This species pair provides an ideal opportunity to investigate the mechanisms and genetic architecture of differential gene flow across a permeable species boundary. In this paper, we present our genomic approaches to identify the underlying genomic regions and song genes that differentiate *L. kohalensis*, one of the pair, and its closest known but allopatric relative, *L. paranigra*. We present a model of the expression of cricket song to assist in our goal to identify the actual genes involved. Once the genomics of song have been identified, differential patterns of gene flow between sympatric species can be approached.

Genomic approaches to identify song genes in Laupala

Numerous genetic methods are available to identify the underlying genetic basis of complex phenotypes. These methods can be broadly grouped into one of three categories: genome analyses, transcriptome analyses, and

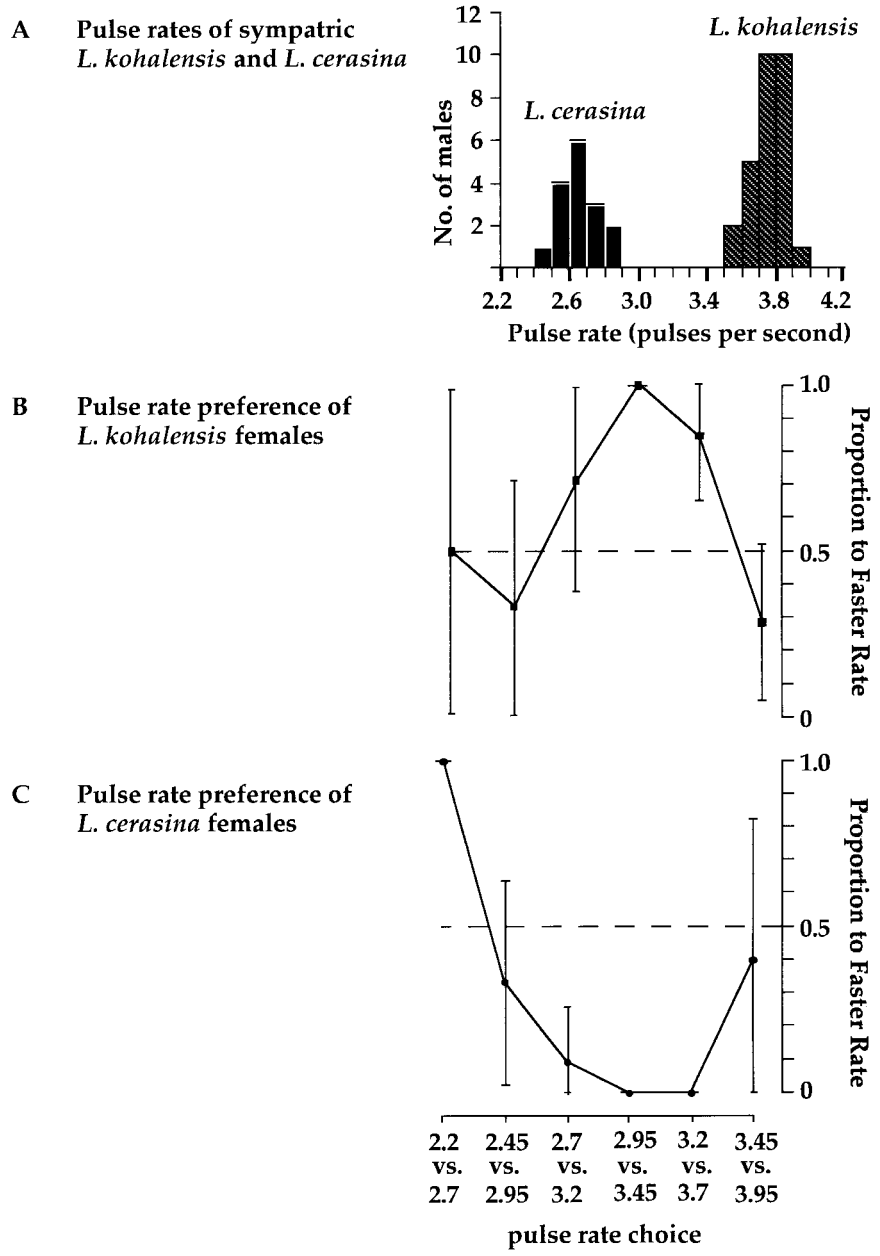

Fig. 2. (**A**) Songs sampled from males collected from the Pololu Valley region of the Kohala Mountains, Hawaii, recorded in the laboratory at approximately 20 °C. The distribution of pulse rate observations for *L. cerasina* and *L. kohalensis* are shown. (After Mendelson and Shaw, 2002). (**B**) Proportion of *L. cerasina* and (**C**) *L. kohalensis* females responding to the faster stimulus of the total that responded for a given trial for each of six phonotaxis trials. Response bias estimates are bounded by ± 1.96 standard deviations (expressed as a percentage), representing a 95% confidence region. The horizontal axis shows the pulse rate trials in pulses per second. (After Mendelson and Shaw, 2002.)

candidate gene analyses (Streelman and Kocher, 2000). Each method can be extremely powerful, especially in genetic model organisms. Combining approaches, however, can provide additional power for identifying the genetic underpinnings of complex phenotypes in natural populations (Streelman and Kocher, 2000; Wayne and McIntrye, 2002; Darvasi, 2003). To map and identify song genes in *Laupala*, we are combining a genomic approach, quantitative trait locus (QTL) mapping, and a transcriptome method DNA microarrays, to generate a short list of candidate genes.

Interspecies hybrids and quantitative genetics of song variation

Classical quantitative genetic methods can yield insight into the number of genes influencing a complex phenotype. Inbred lines or closely related species that possess a distinctly disparate quantitative trait are crossed to produce a hybrid population. An analysis of the segregating phenotypic variation in the hybrid generation can be used to estimate the number of genetic factors influencing a trait. Our studies have shown that the

pulse rates of *Laupala kohalensis* and *L. paranigra* differ by approximately 25 standard deviations (Fig. 1; Shaw, 1996). Songs are easily obtained by recording males individually with a cassette recorder. We digitize these songs, take measurements on the pulse period (Fig. 1) and by taking its inverse, obtain individual pulse rates. Hybrids between *L. kohalensis* and *L. paranigra* can be obtained reliably in both directions in the laboratory, producing F1 males that are intermediate in pulse rate. Using biometrical methods (Lande, 1981), segregation patterns in the F2 and backcross generations indicate a polygenic basis of at least eight genetic factors underlying the pulse rate difference between these species (Shaw, 1996). Thus, a complex genetic basis appears to underlie the simple pulse rate difference that sets these two species apart. However, biometrical methods cannot provide a detailed picture of the genetic architecture of trait variation.

Quantitative trait locus mapping of song variation

QTL analysis identifies chromosomal regions that cosegregate with phenotypic variation and can thus further our understanding of the number, magnitude of effect and genomic location of particular genetic factors. Hybrid progeny are both phenotyped for the trait and genotyped at a large number of genetic markers. A genetic linkage map is estimated and the expression of the phenotype is correlated with the genotype of the genetic markers (Paterson et al., 1988; Tanksley, 1993). From this analysis, the genetic architecture of the trait can be described. In addition, sophisticated analytical procedures can localize regions of the genome which contain the genes that influence variation in the phenotype.

The generation of a male F2 hybrid population has allowed us to produce a genetic linkage map for *L. paranigra* and *L. kohalensis* (Parsons and Shaw, 2002). We generated a genetic linkage map using the amplified fragment length polymorphism (AFLP) technique with either an *Eco*RI/*Mse*I or *Eco*RI/*Pst*I restriction enzyme combination to generate molecular markers that differ between the two species (Parsons and Shaw, 2002). Mendelian segregation patterns indicative of autosomal inheritance as well as X-chromosomal inheritance were mapped using MAPMAKER/Exp 3.00 (Lincoln et al., 1992) to estimate relative positions of autosomal as well as sex chromosome markers. While AFLP markers are largely dominant, we have also identified 17 species-specific alleles based on length variation, giving a small sample of codominant markers. In addition, markers mapped to the X chromosome are hemizygous in the F2 hybrid population because males are XO. We are using this linkage map to estimate genomic regions that cosegregate with pulse rate variation in the F2 mapping population. A preliminary QTL analysis (Shaw and Parsons, 2002) was conducted using interval mapping in QTL Cartographer (Basten et al., 1994–2000). We identified 12 putative QTL localized to multiple linkage groups. Three of these were localized to common regions in *L. paranigra* and *L. kohalensis*, while four and five QTL were specific to *L. paranigra* and *L. kohalensis*, respectively. Those QTL associated with codominant marker loci show relatively small magnitudes of effect (< 10% of the phenotypic difference; Shaw and Parsons, 2002). These results confirm that the genetic basis to song variation is highly polygenic, and suggests small contributions of individual genes to song differences between species.

On its own, QTL mapping will not identify specific genes influencing pulse rate. In *Laupala*, localizing a QTL to a 1 cM interval would include approximately 815 kb of DNA sequence (Parsons and Shaw, 2002), potentially overlapping with hundreds of genes. To demonstrate the involvement of specific genes, we describe our approach using gene expression analysis. Below, we first describe a model of gene expression, which we then use to establish an experimental design to identify the gene products associated with the construction of the neural circuit responsible for stridulation in *Laupala* cricket song.

A model of the molecular expression of cricket song

Gene expression studies rely on a fairly detailed understanding of the tissue and developmental stage in which phenotypic differentiation occurs; the appropriate tissue must be sampled at the appropriate developmental stage in order for meaningful differences in gene expression to be detected. Fortunately, cricket song has been a neurological model system for over 50 years. As a result, a great deal is known concerning the physiological and developmental processes necessary to generate cricket songs.

Crickets sing, or stridulate, by striking a specialized forewing structure, the plectrum (or scraper) against the file (a modified vein), at regular time intervals. A single sound pulse is generated by a single wing closure as the scraper transverses the file. The timing of pulses within a song of a given species is highly stereotyped, and in all species of *Laupala* consists only of a simple train of pulses without any higher order temporal structure (Fig. 1). The precise spatial and temporal patterning of this behavior attracted early neuroethologists (Huber, 1952) and, as a result, stridulation in crickets has become one of the most comprehensively studied rhythmic behaviors in insects.

Cricket stridulation is a neurological phenomenon under plurisegmental control (Huber et al., 1989). Activation centers, which stimulate song production, are located in the mushroom bodies of the supraesophageal

ganglion (Huber, 1964; reviewed by Elsner, 1994). These centers receive and interpret sensory information from the eyes, cerci, antennae, and other sensory areas and determine the environmental appropriateness of singing behavior. Command interneurons descend through the subesophageal ganglion and excite the thoracic central pattern (Hedwig, 2000). Evidence suggests that the stridulation network is located in the meso- and metathoracic ganglion (Huber, 1964; Hennig and Otto, 1996), controlling the rate at which the wing opener and closer motor neurons are excited, thus determining the pulse rate. Additional evidence suggests that ascending interneurons originating in the metathoracic and abdominal ganglia may also contribute to the stridulatory rhythm (Kutsch and Otto, 1972).

The construction of the stridulatory network is under strict genetic control. Unlike birds, crickets are capable of producing species-specific calls in the absence of sensory information either during development or as adults. Deafened crickets and crickets with deafferentiated nerve cords (Bentley, 1969) will produce species-appropriate stridulatory rhythms. Hybridization experiments, such as discussed above for *Laupala*, confirm that songs are under the genetic control of a large number of genes (Bentley and Hoy, 1972; Shaw, 1996).

The developmental origin of the patterned wing movement is also well known (Bentley and Hoy, 1970). The capacity to coordinate wing movements is formed over the course of post-embryonic, rather than embryonic, development. The critical developmental period primarily spans the final three instars during which components of the motor pattern common to both flight and stridulation are expressed in an ordered sequence. Nymphs four molts from adulthood are capable of producing patterned neurological activity in short bursts. This activity is limited, however, and occurs only in the wing depressor neurons. In nymphs three molts from adulthood, the hind wing depressor neurons are capable of more sustained bursts. In this nymphal stage, bursts also begin to show patterned activity characteristic of coordinated wing movements. In the penultimate instar, long trains of spikes can be observed in the hind wing depressor muscle and patterned activity is observed in an elevator muscle. However, depressor and elevator neuronal activity does not exhibit the phase lag necessary for coordinated movement. In the final instar, all motor neurons related to coordinated wing movements are active and fire in the correct phase. During this stage, fully functional stridulation, complete with species-appropriate rhythms, can be elicited from lesioned individuals. This suggests that the stridulatory network is formed prior to the final molt but is actively inhibited until reproductive maturity (Bentley and Hoy, 1970).

Based on these insights, as well as comparative studies of neural circuit evolution, a model of the molecular mechanism of song circuit evolution can be proposed. The model consists of four principal components which we justify below.

1. Changes in the pulse rate have resulted from the modification of interneurons in the central nervous system rather than the modification of sensory or motor neurons.
3. Changes in song patterns result from changes in the cellular properties of the neural circuit rather than a modification of the circuit's architecture.
4. Changes in cellular properties result from alternative patterns of gene expression rather than genetic changes that alter amino acid sequences.
5. Changes in gene expression are most pronounced during development. Differential patterns of gene expression may persist through adulthood, but differences in gene expression at this stage will be less dramatic and may be difficult to detect.

Three principal categories of neurological change can elicit a modification of behavior: sensory systems can change, elements of the central nervous system, which process sensory information and dictate responses, can be altered, and peripheral structures, such as motor neurons and muscles, can be changed to produce a modified response to a central command (Katz and Harris-Warrick, 1999). Neural elements are highly conserved across very divergent taxa (Reichert and Boyan, 1997; Comer and Robertson, 2001), particularly when structures perform identical functions across closely related groups. While the manipulation of sensory information may initiate or inhibit the performance of stridulation, sensory cues appear not to have an influence on song characteristics. The possible exception to this observation, the effect of temperature, is more rightly considered a neurophysiological phenomenon rather than a sensory phenomenon (Pires and Hoy, 1992). Likewise, motor neurons involved in wing movement are highly conserved across orthopterans (Elsner, 1994) and are likely conserved within *Laupala*. Consequently, central processing or command interneurons have likely evolved to produce the observed diversity of pulse rates in *Laupala*.

The central pattern generator responsible for pulse rate could be modified through a number of mechanisms (Getting, 1988): the number of cells in the circuit could change, the pattern of connectivity could be altered, the synaptic properties of the cells could be modified or the conductive properties of the cells' membranes could change. Given the highly conservative nature of neural circuits (Reichert and Boyan, 1997; Comer and Robertson, 2001) and the close phylogenetic relationships of crickets within *Laupala* (Shaw, 2002), it seems unlikely that either the number of cells in the circuit would change or the pattern of connectivity within the circuit would be altered (for a similar example in stick insect

predator evasion, see Büschges and Wolf, 1995). If the song circuit was radically different across species, non-expression or arrhythmic phenotypes might be expected in the hybrids rather than the observed differences in pulse rates (Shaw, 1996). More likely, synaptic strengths (e.g., by varying post-synaptic excitation or inhibition) and/or cellular conductances (e.g., by varying the density of ion channels, thereby altering the speed of impulses through the neuron) have been altered to produce the diversity of pulse rates observed in *Laupala*.

The evolutionary diversification of phenotypes among closely related taxa is often the product of differential patterns of gene expression rather than the modification of protein sequences (Orr and Presgraves, 2000; Davidson, 2001). This is likely to be particularly true for proteins that function in the nervous system. The nervous system tends to exhibit a high degree of parsimony (Comer and Robertson, 2001), in that the same neural machinery is used in multiple contexts (Marder and Calabrese, 1996). This appears to be true with respect to the orthopteran flight system (Comer and Robertson, 2001). Individual gene products are often used in multiple cells, and individual neural circuits are used to produce multiple motor patterns. While the stridulatory circuit in crickets is likely dedicated to the production of only this behavior (Hennig, 1990), it is unlikely that a single gene's product functions in the "song" circuit and nowhere else in the nervous system. As a result, proteins expressed in this circuit must maintain their functionality in a large number of distinct neurons. This constraint should limit the differentiation of proteins expressed in the nervous tissue. Gene expression, in contrast, can be manipulated in a cell-specific manner without threatening the functionality of the nervous system as a whole (Bohm et al., 2000). Thus, patterns of gene expression are relatively unconstrained compared to protein sequence and can evolve rapidly. The rapid differentiation of patterns of gene expression within the cells comprising the "song" circuit is likely to account for the observed diversity of pulse rates within *Laupala*.

Orthopterans exhibit a large degree of post-embryonic neurological development (Chiba et al., 1988; Comer and Robertson, 2001). This has been documented at both the behavioral (Bentley and Hoy, 1970) and cellular level (Cayre et al., 2000). Furthermore, research clearly indicates that the construction of neural circuits responsible for patterned wing movements occurs during the final 3 instars of the cricket post-embryonic development (Bentley and Hoy, 1970). It is during this period, when the circuit is being constructed, that differential patterns of gene expression are likely to be most pronounced. Neurological gene expression continues throughout adulthood and in some instances, can reshape and modify behavioral patterns based on external cues (such as experience; Chiba et al., 1988). However, adult events appear to have no influence on cricket song. Once the "song" circuit is established in the juvenile nervous system, it appears not to be further modified as an adult. Continuous gene expression in the adult is likely necessary to maintain cellular properties of the neurons within the circuit, but such "maintenance" patterns of expression are less likely to contribute to the differentiation of the circuit across species.

Using this model, we have constructed a research program to investigate the developmental patterns of gene expression in the neural centers that control song in *Laupala*. Gene expression will be examined through the use of DNA microarrays. We report the work completed thus far in creating the microarray resources. We have generated a subtracted cDNA library, enriched from neurological cDNAs, that will be spotted on the microarray.

Materials and methods

Gene expression studies of song in Laupala

DNA microarrays consist of thousands of probes to known genes spotted on a glass slide. mRNA from target tissues are reverse transcribed, differentially labeled with a fluorescent dye, and hybridized to the microarray probes. A scanning confocal microscope measures the fluorescent intensity of each labeled cDNA at each spot. Computer analysis calculates the relative intensity of each labeled cDNA to calculate the relative level of gene expression of each tissue for each of the genes spotted on the array. Ultimately, we are interested in identifying those genes related to the diversification of cricket song in *Laupala*. Gene expression studies have the advantage, relative to genomic approaches, that the expression of divergent phenotypes is correlated directly to the relative abundance of an expressed gene product rather than to anonymous, albeit localized, regions of the genome. The strength of microarray studies lies in their ability to examine thousands of genes simultaneously and to directly link the differentiation of phenotypic characters with specific genes.

Library construction

Two subtracted (brain transcripts – body transcripts) cDNA libraries were constructed. One was developed from adult tissues. The second library was generated from juvenile tissue. While no precise developmental information is available for this species, based on the position and shape of the wing buds all juvenile individuals appeared to be in their third instar prior to adulthood. To construct the adult nervous tissue library, the ventral nerve cords of three adults were surgically re-

moved. RNA was collected, using the methods described below, from all three adult nerve cords and from one adult body that had had its nerve cord removed. The nerve cords of five juveniles were surgically removed. RNA was extracted from the pooled nerve cords as well as from each body that had had its nerve cord removed. All individuals were anesthetized with carbon dioxide and their nerve cord surgically removed while submersed in RNA*later* (Ambion). Both the bodies and the excised nerve cords were preserved in RNA*later* and stored at –80 °C prior to RNA extraction.

The tissues were pulverized with a micropestile in a 1.5 ml tube. Total RNA was extracted using either SV Total RNA Isolation Kit (Promega) (adult) or RNeasy Mini Kit (Qiagen) (juvenile) following the manufacturer's instructions. cDNA construction and subtraction loosely followed the methods of Diatchenko et al. (1996) as implemented in the Clontech PCR-Select cDNA subtraction kit. Briefly, single stranded cDNA was constructed via a poly T primer using Clontech Powerscript reverse transcriptase. Double stranded cDNA was constructed and amplified. The pooled "body" cDNA was digested overnight with *Rsa*I (NE Biolabs). The nervous tissue cDNA was ligated to two adaptors. In one reaction, nervous tissue cDNA was ligated to Clontech PCR-Select cDNA subtraction kit adaptor 1. In a separate reaction, nervous tissue cDNA was ligated to Clontech PCR-Select cDNA subtraction kit adaptor 2. Both ligation reactions incubated overnight at 16 °C.

The normalization and subtraction of cDNAs common to both tissue sets (body and nervous tissue) from the pool of nervous tissue cDNAs was achieved through two successive hybridizations (Fig. 3). During first hybridization, the digested "body" cDNA was hybridized in molar excess to either the nervous tissue-adaptor 1 or the nervous tissue-adaptor 2 cDNA populations. This process forced cDNAs derived from nervous tissue to hybridize to its compliment cDNA derived from body tissue. Such constructs will not geometrically amplify during subsequent PCR steps since the "body" strand of the cDNA lacks the necessary primer annealing sites (Clontech Adaptor 1 or Adaptor 2). During the second hybridization, the products of the first hybridization were pooled and additional denatured "body" cDNA was added. This hybridization, in addition to allowing more cDNAs common to both body and nervous tissue to hybridize, allows complementary nervous tissue cDNAs ligated to alternative adaptors to hybridize. This process generates cDNAs specific to the nervous tissue with appropriate priming sites at each end of the cDNA. Two rounds of suppressive PCR were used to enrich the population of cDNAs found predominantly in nervous tissue.

The purified cDNAs were TA using the TOPO TA cloning kit (Invitrogen). Individual colonies were picked from overnight cultures, isolated, grown overnight in liquid LB-AMP medium and plasmid DNA harvested using a miniprep kit (Qiagen). The harvested plasmid DNA was diluted 1:10 and cycle sequenced. The DNA sequences were edited by eye and vector sequence was

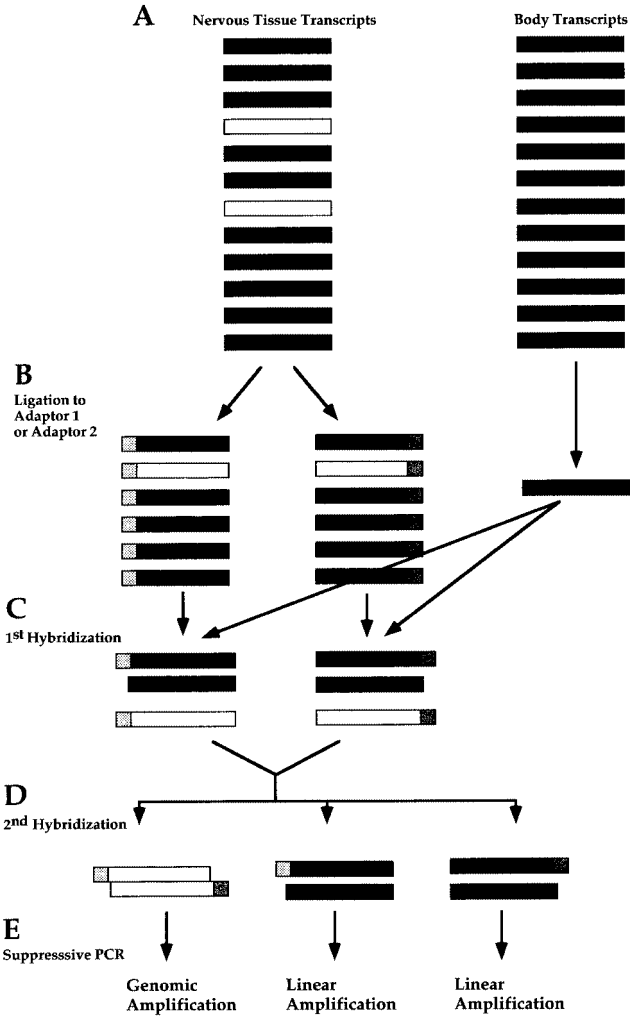

Fig. 3. The enrichment for nervous tissue specific cDNAs. (**A**) Double stranded cDNA is constructed from mRNA collected from nervous tissue and from the remainder of the body. cDNAs specific to the nervous tissue are diagrammed in white. cDNAs common to both the nervous tissue and the remainder of the body are diagrammed in black. (**B**) cDNAs derived from nervous tissue are split and two adaptor ligation reactions are performed. One reaction ligates Adaptor 1 to each cDNA. The other reaction ligates Adaptor 2 to each nervous tissue-derived cDNA. The cDNAs derived from body tissues are digested with RSAI. (**C**) The 1st hybridization. The two pools of adaptor-ligated nervous tissue cDNAs are separately hybridized to the digested body cDNA. (**D**) The 2nd hybridization. Both pools of adaptor-ligated nervous tissue cDNAs are combined and allowed to hybridize with each other as well as with additional digested body cDNA. (**E**) Suppressive PCR amplifies only those cDNA products which are ligated to both Adaptor 1 and Adaptor 2, thereby selectively amplifying only nervous tissue-derived cDNA.

stripped using Sequencher. Putative Expressed Sequence Tag (EST) identity was established through comparisons with known sequences in the National Center for Biotechnology Information (NCBI) nonredundant (nr) database using the tblastx algorithm. Putative identities were assigned if the E (the E value is roughly the probability that a sequence in the database will match a given sequence by chance) and Identity value were less than 1 and greater than 40%, respectively, for a given comparison.

Results

Two cDNA libraries were constructed; one derived from adult tissue and a second one derived from juvenile tissue (Table 1). Ninety-two clones were sequenced from the adult library. Among these, 43 (46%) unique ESTs were identified. An EST was defined as unique if the sequence of the clone could not be aligned with any other clone sequence in the library. The length of the ESTs in the adult library ranged from 91 to 927 bp. An individual EST, in some instances, consisted of the consensus sequence of multiple sequenced inserts. The average size of the ESTs in the adult library was approximately 414 bp. Sixty-three clones were sequenced from the juvenile library. Of these, 56 (88%) represented unique ESTs. The length of the ESTs in the juvenile library ranged from 112 to 751 bp. The average EST size was approximately 379 bp. Interestingly, redundancy is greatly reduced in the juvenile library relative to the adult library. This suggests, as one might expect, that the diversity of expressed genes is higher in juvenile tissue than in adult tissue.

The ESTs in each library could be divided into three categories (Table 2).

- ESTs which represent novel sequences. Novel sequences were defined as sequences that could not be aligned to a sequence within the NCBI non-redundant database with an E value less than one and an Identity value greater than 40%. Ten ESTs from the adult library were observed in this category; 37 ESTs from the juvenile library fell into the novel sequence library.
- The second category consisted of ESTs in which a similar sequence was identified in the database but no putative function could be assigned. Four and eleven ESTs were observed in this category from the adult and juvenile libraries, respectively.
- The final category consisted of ESTs whose sequence was similar to a sequence in the database and for which a putative function could be assigned. Thirty adult ESTs had putative functions assigned to them. Putative functions were assigned to eight juvenile ESTs.

A consideration of the distribution of ESTs across these three categories in both libraries reveals an interesting observation. The juvenile library has nearly four times as many novel ESTs as the adult library and more than a third fewer ESTs with a putative function. This suggests that juvenile neurological tissues contain transcripts of genes specific to either *Laupala*, crickets, and/or orthopterans (all of which are underrepresented in the NCBI database).

Among the adult sequences with putative function, several ESTs were of interest (Table 3). Several putative chemosensory (3) and hemolymph (7) proteins have been identified. A *Laupala* opsin is putatively identified. Only one other orthopteran opsin has been thus far described (locust, Towner et al. 1997). No cricket opsin

Table 1. Characteristics of adult and juvenile EST libraries.

	Adult Library	Juvenile Library
No. clones sequenced	92	63
Unique ESTs	43 (46%)	56 (88%)
Size Range	91–927 bp	112–751 bp
Average	414 bp	379 bp

Table 2. Putative gene homologies in EST libraries. Novel sequences were defined as those EST sequences which could not be aligned with sequences within the NCBI non-redundant database with an E value less than 1 and an identity value greater than 40%.

	Adult Library	Juvenile Library
Novel Sequence	10	37
Similar Sequence (unknown function)	4	11
Similar Sequence (putative function)	30	8

Table 3. Putative identity of ESTs within the adult library. Representation refers to the frequency of ESTs within the library which aligned to the given sequence.

Adult Library	
Similar Sequence	Representation
Hemolymph	7
Opsin	5
Chemosensory	3
K^+ channel (Shaker)	1
CAM Kinase	1
Ecdysone Receptor	1
Retinaldehyde Binding Protein	1

has been previously sequenced. A putative homologue to the *Drosophila* potassium channel "Shaker" has been identified as well as a putative CaM kinase. CaM kinase phosphorylates the synaptic vesicle protein synapsin during the release of neurotransmitters into the synaptic cleft. No ion channel or CaM kinase has been previously described for an orthopteran.

Additional interesting ESTs with putative functions were identified in the juvenile library (Table 4). Three ESTs were similar to transcripts derived from human placental tissue. While their exact functions are unknown, the high concentration of ion channels in the human placenta suggests that these ESTs may code for *Laupala* ion channels. One EST was similar to a transcript derived from the developing brain of a mouse. Again, its precise function is unknown, but given that little is known concerning the genetic processes involved in the development of orthopteran nervous tissue, such an EST may yield invaluable insights. Another EST was similar to a gene involved in a human developmental disorder (Werner syndrome which causes premature aging). The study of the gene that produced this EST may not only shed light upon the developmental process in crickets, but may also provide insight into the general activity of this gene and its role in Werner Syndrome. An EST with significant sequence similarity to synapsin was also identified. This is the first synapsin to be identified in an orthopteran.

In addition to comparing the EST sequences from both libraries to sequences deposited in the NCBI database, EST sequences were compared across libraries. While the within library level of redundancy was sufficient to detect multiple copies of individual ESTs, cross library redundancy was not detected. Rather, adult and juvenile EST collections represent distinct sets of expressed genes. While it is highly unlikely that adult and juvenile expressed genes are mutually exclusive, the observed pattern suggests that significant changes in gene expression occur during the ontogeny of the *Laupala* nervous system.

Table 4. Putative identity of ESTs within the juvenile library.

Juvenile Library	
Similar Sequence	Representation
Synapsin 2B	1
Human Placental	3
Human Developmental Disorder (Werner, Ellis van Creveld)	1 (2)
Human Skin Cancer	1
Mouse Developing Brain	1
Immunoglobulin	1

Discussion

The genetic architecture underlying divergence has vital consequences for many speciation models (e.g., see discussions in Barton and Charlesworth, 1984; Carson and Templeton, 1984). We also expect that genetic signatures of divergence will inform us about processes of speciation (Coyne and Orr, 1998; Shaw and Parsons, 2002). Recent attention on the permeable nature of species boundaries also begs an understanding of the genetic architecture of speciation phenotypes, sharing a focus with students of hybrid zones (Barton and Hewitt, 1985). All of these areas lead to the question of what genetic changes cause speciation. The study of porous species boundaries demands close scrutiny of the speciation phenotypes upon which selection acts to drive populations apart, given that gene flow between species will work to homogenize populations.

Song variation among closely related acoustic insects is appreciated to assist in reducing gene flow between diverging forms (reviewed in Wells and Henry, 1998). The taxon of Hawaiian Trigonidiinae, conspicuously characterized by song differences and represented by over 160 species is the result of frequent cladogenesis involving divergence in acoustic signaling. Most of these species are morphologically cryptic, with only 10 species formally recognized before 1994 (Otte, 1994). In crickets, undertaking studies to determine the genetic basis of song diversity is ideal because of the rich history of neurophysiological research (Huber et al., 1989), the functional context in which song is used (Alexander, 1962; Huber and Thorson, 1985; Stabel et al., 1989), song's distinctiveness among species and importance in speciation (e.g., Otte, 1994), and the macroevolutionary consequences of song evolution (Otte, 1992). In the endemic genus *Laupala*, the question is focused more finely because only a single axis of diversification, the pulse rate of the male calling song, differs repeatedly among species. Many examples of sympatric *Laupala* communities exist in the understory of Hawaiian mid-elevation rainforests, where species are acoustically distinct but otherwise are ecologically and reproductively similar.

We have been studying one community on the Big Island of Hawaii where *L. kohalensis* lives sympatrically with *L. cerasina*. These species have exchanged mtDNA and possibly nuclear genes (Shaw, 2002; Mendelson and Shaw, 2002) but do not readily hybridize in the laboratory. Nonetheless, *L. kohalensis* can be crossed to its closer, allopatric relative, *L. paranigra* (Fig. 1; Shaw, 1996), and genomic regions identified through studies between *L. kohalensis* and *L. paranigra* can be used to examine the genomic interactions between the sympatric *L. kohalensis* and *L. cerasina*.

Our biometrical and QTL studies have shown that despite the simple pulse rate difference between *L. kohalensis* and *L. paranigra* (Fig. 1), multiple genes, with apparently small effects (Shaw, 1996; Shaw and Parsons, 2002) located in several linkage groups underlie variation between species. Furthermore, song is deployed in a complex behavioral interaction with females who express acoustic preferences (Shaw, 2000; Shaw and Herlihy, 2000; Mendelson and Shaw, 2002). Our ability to predict genomic regions free to flow between *L. kohalensis* and *L. cerasina* will benefit by understanding the series of interactions at the behavioral, neurophysiological and genetic levels, and the associated selection intensities and linkage relationships influencing the underlying song genes.

Building on existing knowledge about the neural substrates controlling the production of song, we present the first molecular model of cricket song variation. We use this model to inform our study of gene expression, which is designed to identify candidate genes underlying song expression and variation. We focus on the likelihood of expression differences between juvenile and adult, spanning a developmental period when the neuromuscular song apparatus is laid down. Several results are encouraging at this preliminary stage. First, we have identified putative *Laupala* homologues to genes in other organisms where function is known or implicated (Table 3), including neurologically relevant transcripts that indicate a successful normalization enriched for nervous tissue genes. Our identification of homologues from other organisms will provide a functional context in which to interpret expression differences between species that differ in song. We also have tentative evidence that patterns of gene expression have changed between juvenile and adult in the nerve cord (Tables 3, 4). While this is expected, it does indicate that extensive gene activity is occurring in the cricket nerve cord at the point in development when neurophysiological studies indicate cricket stridulation becomes functional. In addition, while our study is focused on song, we have also found several interesting transcript products previously not known from the order Orthoptera.

Our approach to identifying genomic regions carrying song genes, and song genes themselves, involves both QTL mapping and gene expression studies, which in combination will provide crucial perspectives on species boundaries and the genetics of speciation. QTL results identify linkage groups that likely consist of hundreds of genes, but nonetheless can act as units of introgression across species boundaries (Rieseberg et al., 1995; Rieseberg et al., 1999). A microarray project will identify changes in gene expression contributing to the diversification of calling song, however, "key" changes are not easily distinguished from correlative changes in gene expression. The overlap of these two data sets, and the identification of genes with altered patterns of gene expression which map to regions of the genome within a significant QTL, is a powerful approach to identify, localize, and estimate the effect of genes influencing cricket song.

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