Nuclear Gene Genealogies Reveal Historical, Demographic and Selective Factors Associated With Speciation in Field Crickets

Richard E. Broughton and Richard G. Harrison

Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853

Manuscript received June 5, 2002
Accepted for publication January 8, 2003

ABSTRACT

Population genetics theory predicts that genetic drift should eliminate shared polymorphism, leading to monophyly or exclusivity of populations, when the elapsed time between lineage-splitting events is large relative to effective population size. We examined patterns of nucleotide variation in introns at four nuclear loci to relate processes affecting the history of genes to patterns of divergence among natural populations and species. Ancestral polymorphisms were shared among three recognized species, Gryllus firmus, G. pennsylvanicus, and G. ovisopis, and genealogical patterns suggest that successive speciation events occurred recently and rapidly relative to effective population size. High levels of shared polymorphism among these morphologically, behaviorally, and ecologically distinct species indicate that only a small fraction of the genome needs to become differentiated for speciation to occur. Among the four nuclear gene loci there was a 10-fold range in nucleotide diversity, and patterns of polymorphism and divergence suggest that natural selection has acted to maintain or eliminate variation at some loci. While nuclear gene genealogies may have limited applications in phylogeography or other approaches dependent on population monophyly, they provide important insights into the historical, demographic, and selective forces that shape speciation.

PHYLOGENIES of alleles or haplotypes (gene genealogies) can reveal the sequence and timing of speciation (lineage-splitting) events and the geographic context in which they occurred (Hudson 1990; Templeton 1998; Wakeley and Hey 1998; Kliman et al. 2000). However, random sorting of ancestral polymorphisms, hybridization and introgression, and natural selection obscure the signature of previous history; therefore, gene trees and species trees may not be congruent (Neigel and Avise 1986; Pamilo and Nei 1988; Takahata 1989; Wu 1991; Hudson 1992; Nichols 2001). For any single locus, inferences of recent evolutionary history are most reliable if coalescence times for samples of alleles are less than the time between lineage-splitting events. Under these circumstances, diverging lineages become exclusive groups (groups in which all members are more closely related to each other than to any individual outside of the group) before further lineage-splitting events occur.

Population bottlenecks or persistent small $N_e$ will reduce coalescence times for all loci; diverging lineages will become exclusive groups more quickly. Fixation of new variants via directional selection (“selective sweeps”) will also reduce coalescence times, but only for markers tightly linked to the selected locus. In contrast, balancing selection promotes the maintenance of polymorphism and thus increases coalescence times; lineages become exclusive groups more slowly. Of course, the footprint of natural selection in the genome depends on the strength of selection and the local recombination rate; if selection is strong and recombination rates are low, then a selective sweep may carry to fixation a relatively large genomic region (Kaplan et al. 1989), and variation at linked markers will reflect the fixation event. Weak selection and high rates of recombination mean that only a very small region will be affected by a selective sweep or by balancing selection (Ting et al. 2000).

The relative merits of nuclear vs. mitochondrial loci for evolutionary studies have been much discussed (e.g., Wu 1991; Moore 1995; Hoelzer 1997; Avise 2000; Hare 2001). That coalescence times for mitochondrial genes will be only one-quarter those for nuclear genes (Birky 1991) is an argument in favor of using such genes for inferring the sequence of recent lineage-splitting events (Moore 1995). However, the absence of recombination in mtDNA means that a selective sweep acting anywhere in the mitochondrial genome will carry a single haplotype to fixation, reducing variation across the entire genome. Amounts of variation will then no longer reflect historical effective population sizes, and information on population history is lost.

Among nuclear genes, there is also considerable variation with respect to the probability that they will provide clear resolution of past lineage-splitting events. Both
persistent ancestral polymorphisms and introgression subsequent to secondary contact may produce nuclear gene genealogies that are incongruent with each other and with relationships defined by morphology. For divergent lineages in which barriers to gene exchange are incomplete, boundaries are often semipermeable or porous (Harrison 1991; Wu 2001; Machado et al. 2002). That is, the genetic architecture of reproductive isolation has important consequences for patterns of differentiation and introgression. Therefore, gene genealogies for closely related “species” will vary with gene region (see Hey 1994; Wang et al. 1997; Ting et al. 2000), and in regions closely linked to genes that have experienced recent selective sweeps and/or genes that contribute to reproductive isolation, genealogies will more likely reveal the two forms as exclusive groups (Ting et al. 2000).

Comparative studies of gene genealogies for multiple loci are still not common in nonmodel organisms, and therefore the data to test the ideas discussed above are few. Here we analyze sequence variation in introns of four nuclear gene loci in North American field crickets (genus Gryllus) and compare these data with already published data on mtDNA sequence variation in these insects. Our goal is to elucidate genealogical patterns within and among this group of closely related species and to explain observed patterns in terms of a recent history of demographic change and natural selection.

North American crickets in the genus Gryllus are a group of closely related species that have been well characterized morphologically, behaviorally, ecologically, and genetically (Alexander 1957, 1968; Harrison 1979, 1983, 1986; Rand and Harrison 1989; Harrison and Bogdanowicz 1995, 1997; Willett et al. 1997). From 1915 until 1957 all field crickets in North America were considered to be a single species, on the basis of Rehn and Heb ard’s (1915) assessment that different morphological forms represented the adaptation of a very plastic species to local environmental conditions. Although morphologically similar, the crickets have clearly diverged in ecology (habitat, life cycle) and behavior (calling song), and variation in these traits formed the initial basis for recognizing multiple independent lineages (Alexander 1957). The most distinct mtDNA haplotypes among those sampled from all of the North American species are only 6.8% (Huang et al. 2000) or 8% (Harrison and Bogdanowicz 1995) divergent, suggesting recent (Pliocene/Pleistocene) diversification.

MtDNA restriction site and sequence data indicate that the three egg-overwintering species (Gryllus pennsylvanicus, G. firmus, and G. ovisopis) are all very closely related (sequence divergence of <1%). G. ovisopis is found in woodlands in northern Florida and southern Georgia. Both G. pennsylvanicus and G. firmus are widespread in disturbed habitats. They overlap and hybridize along an extensive zone that runs from southern Connecticut, across the Hudson Valley, and then along the eastern front of the Appalachian and Blue Ridge Mountains (Harrison and Arnold 1982). G. pennsylvanicus is found in inland and upland localities while G. firmus is found along the coastal plain. Although individuals of mixed ancestry are found in the hybrid zone, allele frequency differences at three allozyme loci and diagnostic haplotypes for mtDNA and anonymous nuclear restriction fragment length polymorphisms (RFLPs) are characteristic of allopatric populations (Harrison 1986; Harrison et al. 1987; Rand and Harrison 1989; Harrison and Bogdanowicz 1997). MtDNA sequences from the COI-COII region distinguish four clades that correspond to northern and southern populations of the two species. However, relationships among the four clades are not resolved (Willett et al. 1997), and the two species are not clearly exclusive groups for mtDNA. On the basis of cytochrome b sequences, Huang et al. (2000) suggested that G. pennsylvanicus and G. firmus are not even sister species, that G. ovisopis is sister to G. pennsylvanicus. However, they sampled only a single locality and one or two individuals for each species. What is clear is that the three egg-diapausing species have all diverged recently and that G. pennsylvanicus and G. firmus have subsequently come together to form an extensive hybrid zone.

Of the four nuclear gene loci that we examined [calmodulin (Cam), cytochrome c (Cyt-c), elongation factor 1a (Efla), and phosphoglucose isomerase (Pgi)], only Pgi has been characterized previously in field crickets. Allozyme studies revealed a Pgi polymorphism shared among several species (Harrison 1977, 1979; Harrison and Arnold 1982). Not only do G. pennsylvanicus, G. firmus, and G. veletis share the same array of electrophoretic alleles, but in the broadly sympatric (but distantly related) G. pennsylvanicus and G. veletis, the frequency of the most common Pgi electromorph varies in parallel (Harrison 1977). The patterns of shared electrophoretic alleles and parallel variation led to the suggestion that balancing selection may be acting at this enzyme locus. Comparison of DNA sequences in G. pennsylvanicus and G. veletis revealed that electrophoretic mobility classes are heterogeneous, i.e., that the same shifts in electrophoretic mobility have apparently occurred independently both within and between species (Katz and Harrison 1997). This is not consistent with a trans-specific polymorphism maintained by balancing selection. However, the DNA sequence data also suggest that Pgi is not evolving neutrally. Comparisons of patterns of variation at the Pgi locus with those observed for other nuclear genes may shed additional light on the forces acting to maintain the polymorphism.

MATERIALS AND METHODS

Cricket samples: Samples of G. firmus and G. pennsylvanicus were each obtained from three localities in the eastern United
States. Individuals of *G. pennsylvaniaeus* were collected from Ithaca, New York; Sharon, Connecticut; and Harrisonburg, Virginia. *G. firmus* were collected from Guilford, Connecticut; Saybrook Point, Connecticut; and Seaside Park, New Jersey (Figure 1). Collecting sites were chosen on the basis of previous collecting experience, and crickets were identified on the basis of differences in body length, hind wing length, ovipositor length, and tegmina color (Harrison 1986). *G. ovipositis* was obtained from a culture of crickets from Gainesville, Florida and *G. veletis* was collected from Sharon, Connecticut. Crickets were transported alive to the laboratory where they were frozen at −80°C until DNA isolation.

**PCR amplification, cloning, and sequencing:** Sequences of the *Cam, Cyt-c, and Efla* introns were obtained by PCR amplification, using primers located within adjacent exons but near the exon-intron boundaries (Palumbi 1996, p. 240). Primers were selected from the insect nuclear primer set assembled by B. Crespi and obtained from J. Hobbs at the University of British Columbia, Vancouver, Canada (Table 1). For *Pgi*, primers were designed on the basis of sequences in Katz and Harrison (1997) with one primer in an exon and the second within the intron itself to avoid sequencing through a tandem repeat. Sequences of *Cyt-c, Cam, and Efla* were obtained directly from PCR products. Reactions contained 1× Taq buffer (GIBCO/BRL, Gaithersburg, MD), 1.5 mM MgCl₂, 200 μM each dNTP, 0.5 μM each primer, 1 unit Taq polymerase, and ~100 ng template DNA in a 50-μl volume. Cycling parameters were 35 cycles of 94°C for 15 sec, 48°C–54°C for 15 sec, and 72°C for 30–90 sec (~60 sec/kb of product length). Templates were prepared for sequencing with QIAquick PCR product purification columns (Qiagen, Valencia, CA). All sequencing reactions employed the ThermoSequenase ³²P-labeled terminator kit (USB) according to manufacturers’ recommended protocols. Products were separated on manual sequencing gels and visualized with autoradiography. Both strands of each intron were sequenced.

Direct sequencing of PCR products revealed many nucleotide sites at which individuals were heterozygous. Although there are methods for estimating individual haplotypes from population data (e.g., Clark 1990), it is not always possible to fully determine gametic phase without additional experimental data. *Cam* and *Efla* had low levels of heterozygosity and haplotypes could be determined unambiguously. *Cyt-c* had somewhat higher levels of heterozygosity so unknown haplotypes were estimated on the basis of the presence of observed homozygotes. This eliminated most ambiguity in haplotype assignments. High levels of heterozygosity made it particularly difficult to define haplotypes for *Pgi*. In this case, purified PCR products were cloned into the pGEM T-vector (Promega, Madison, WI) and transformed into Escherichia coli DH5α cells. Plasmids were purified via standard alkaline lysis methods and sequenced using universal primers. Typically, two to four clones from each individual cricket were sequenced, allowing gametic phase and allele identities to be determined. Of all the analyses performed, only the genealogical and recombination analyses would be affected by the lack of unambiguous haplotype assignment for *Cyt-c* and even in these cases, the consequences are expected to be slight given that most of the relevant sites are singleton variants.

**Data analysis:** Sequences were aligned with Sequencher (Gene Codes, Ann Arbor, MI) and modified manually. The number of polymorphic sites and their distribution among species was determined. Within species, nucleotide variability was measured using θ, the proportion of polymorphic sites in a sample (Watterson 1975), and π, the average number of nucleotide differences among sequences in a sample (Nei and Li 1979). Tajima’s *D* (Tajima 1989) and Fu and Li’s *D* *₀* (Fu and Li 1993) were calculated to test for departures from neutrality. Tajima’s *D* is based on the expectation that under mutation-drift equilibrium, θ and π should estimate the same parameter.

**TABLE 1**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Efla</em></td>
<td>EF0</td>
<td>TCCGGATGGGCAAGGCAGAATAAG</td>
<td>Villablanca et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>EF2</td>
<td>ATGTCGCCAGTGGCGAAATCCA</td>
<td></td>
</tr>
<tr>
<td><em>Cam</em></td>
<td>Cal-1</td>
<td>GCCGACGTGCAARGAYATGATCA</td>
<td>Duda and Palumbi (1999)</td>
</tr>
<tr>
<td></td>
<td>Cal-2</td>
<td>GTGTCCTTCATTTNCKTGCCAT</td>
<td></td>
</tr>
<tr>
<td><em>Cyt-c</em></td>
<td>Cyt-c-5'</td>
<td>AAGTGTGCYCARTGCCACAC</td>
<td>Palumbi (1996)</td>
</tr>
<tr>
<td></td>
<td>Cyt-c-3'</td>
<td>CATCTGGTGGCGGCGGATGATTCTT</td>
<td></td>
</tr>
<tr>
<td><em>Pgi</em></td>
<td>370+</td>
<td>CCAATACCTACATAGATTGCTG</td>
<td>Katz and Harrison (1997)</td>
</tr>
<tr>
<td></td>
<td>RCMR</td>
<td>GCTGAAAATAATGCTATTTTGAGTCA</td>
<td></td>
</tr>
</tbody>
</table>
4Nμ, so significant differences in these estimates may indicate departures from neutrality. Fu and Li's F* compares differences between estimates of 4Nμ, on the basis of h, the (number of singleton mutations) and S (the number of segregating sites).

The neutral theory also predicts that loci with high polymorphism within species will exhibit high divergence between species and loci with low polymorphism will exhibit low divergence. Thus, if loci are behaving neutrally, the ratio of polymorphism to divergence should be similar for each locus. Polymorphism and divergence were compared between loci using the Hudson-Kreitman-Aguadé (HKA) test (Hudson et al. 1987). Measures of variation and standard HKA tests were performed with the computer program DnaSP version 3.14 (Rozas and Rozas 1999). Multiple-locus HKA tests based on coalescent simulations were performed with the computer program HKA (provided by Jody Hey, Rutgers University). To test for spatial heterogeneity in the ratio of polymorphic sites to fixed differences, we employed the DNA Slider program (McDonald 1998).

Genealogical patterns were visualized via neighbor-joining trees constructed from Jukes-Cantor distances with the computer program MEGA2 (Kumar et al. 2001) and by statistical parsimony networks (Templeton et al. 1992) as implemented by the computer program TCS (Clement et al. 2000). The regions sequenced for at least two of the loci appear to have been subject to recombination (Kaplan and Hudson 1985, see Table 2) so portions of the introns may reflect different historical patterns. We employed a distance-based approach, which groups alleles on the basis of overall similarity; therefore, resulting trees cannot be assumed to represent the true genealogy, but they will reflect approximate patterns of relatedness. The statistical parsimony approach allows reticulations (if necessary) to reflect uncertainty generated by recombination and homoplasy (Templeton et al. 1992). Potential population structure was investigated by comparing pairwise π values (with Jukes-Cantor distances) among sample localities. Significance of these differences was assessed by permutation tests.

Analysis of nucleotide variation with respect to speciation has recently been applied in a hypothesis-testing framework where empirical frequencies of polymorphisms and fixed differences are compared to a null model (the isolation species model) using coalescent simulations (Wang et al. 1997). The isolation species model assumes that variation is neutral and that there has been no gene flow beyond a point in the past at which the species diverged. The fit of the data from G. firmus and G. pennsylvanicus to the isolation model was tested with the program WH (provided by Jody Hey, Rutgers University).

**RESULTS**

**Polymorphism and divergence:** The number of sequences obtained ranged from 34 for Pgi to 40 for Cam (see Figures 2 and 3). Sequences of Cyt-c and Cam were only 298 and 309 bp in length; sequences of Ef1α and Pgi were longer, 616 and 749 bp, respectively. Levels of variation differ substantially among loci (Table 2). For G. firmus and G. pennsylvanicus, polymorphism as measured by θ and π differs by more than an order of magnitude between the two extreme values for Ef1α and Pgi. At each locus, values of θ and π are similar, and neither Tajima’s D nor Fu and Li’s D* differed significantly from the neutral expectation of zero. There were no sites within species with more than two nucleotide states so that S, number of segregating sites, is equal to h, the minimum number of mutations; consequently Fu and Li’s D* is identical to their other statistic, F*.

Although polymorphism varied substantially among loci, levels of variability are similar between the two species G. firmus and G. pennsylvanicus. Levels of polymorphism were not correlated with intron length. The lowest and highest levels of polymorphism were found in the two longest intron sequences (Ef1α and Pgi; see Table 2).

The estimated minimum number of recombination events based on the presence of all four possible recombination products (Table 2) was proportional to the number of segregating sites. Therefore, the number of observed recombination events is related to the number of opportunities to detect recombination and provides no evidence that recombination rates differ among loci.

Interspecific divergence is summarized in Table 3. Divergence between G. firmus and G. pennsylvanicus was uniformly low, although average number of pairwise sequence differences is relatively high for Cyt-c and Pgi because of the large number of haplotypes. There were no fixed differences between these species at any locus and about one-half of all polymorphisms were shared between them (36 across all loci and 25 for Pgi alone), the other sites being polymorphic in one or the other species but not both. There were no fixed differences between G. firmus, G. pennsylvanicus, and G. ovisopis at Ef1α, but variability at this locus was low. The similarity of these species was also reflected in the presence of three polymorphisms shared among all three species (one for Cyt-c and two for Pgi). In contrast, G. veletis has diverged substantially from the other species, consistent with previous phylogenetic analyses based on mtDNA (Harrison and Bogdanowicz 1995; Willett et al. 1997; Huang et al. 2000).

Average pairwise divergence (π) among G. firmus and G. pennsylvanicus sample localities was generally greater for interspecific comparisons than among intraspecific populations. However, in only one case was divergence significant (Pgi for G. firmus Saybrook Point vs. G. pennsylvanicus Harrisonburg) and in that case there were only two haplotypes for the Harrisonburg sample so the test result is suspect. This suggests little population subdivision within species.

**Genealogical patterns:** Neighbor-joining trees for each locus are shown in Figure 4. Because recombination has affected Pgi and Cyt-c (Table 2), we cannot treat the trees as true genealogies in the sense of estimating a single history for each allele; rather these trees are approximations of genealogy based on overall similarity. For each locus, some alleles in one species are at least as similar to alleles in another species as they are to other alleles within the same species. Although haplotypes defined for Cyt-c are somewhat arbitrary, because we could not directly assess gametic phase when heterozy-
gotes had more than one segregating site, alternative haplotype assignments do not change the fundamental characteristics of the gene genealogies. Thus, alleles in *G. firmus* and *G. pennsylvanicus* do not form exclusive groups for any locus. Furthermore, for three of the four loci (all but *Cam*) *G. firmus* and *G. pennsylvanicus* do not form an exclusive group with respect to *G. ovisopis*. This morphism data supports the isolation species model of Wakeley and Hey (1997). Observed counts suggest that coalescence times for *Ef1a/H9251*, *Cyt-c*, and *Pgi* predate the common ancestor of all three species. Coalescence for *Cam* appears to occur after divergence of *G. ovisopis* but before divergence of *G. firmus* and *G. pennsylvanicus*. Although introgression could also result in shared alleles, the geographic distribution of alleles is not consistent with this hypothesis (see below). Networks produced under statistical parsimony (not shown) were consistent with patterns from neighbor-joining trees and it was not possible to draw nonoverlapping clades corresponding to *G. firmus* and *G. pennsylvanicus* for any locus. The genealogies also indicate little geographic structure at the level of individual alleles. In numerous instances, alleles from the same population appeared in divergent portions of a gene network. In addition, alleles from geographically distant localities often cluster together.

**A model of speciation:** We examined the fit of polymorphism data to that predicted by the isolation species model of Wakeley and Hey (1997). Observed counts of sites exhibiting polymorphism shared by two species, polymorphic in species 1 but monomorphic in species 2, polymorphic in species 2 but not in 1, and fixed differences were used to estimate the population parameter for each species (*θ₁* and *θ₂*), the population parameter for the ancestral species (*θₐ*), and the time (*T*) in 2Nₑ generations to the common ancestor. Parameters were as follows: *θ₁* = 9.166, *θ₂* = 7.937, *θₐ* = 18.100 (95% C.I. = 6.84–48.24), and *T* = 0.050 (95% C.I. = 0.0–0.487) (where *G. firmus* is population 1 and *G. pennsylvanicus* is population 2). These results suggest that
the ancestral population was approximately double the size of either descendant population. This is consistent with a speciation scenario in which a large ancestral population becomes subdivided, as would be expected by a vicariance event, and the resulting daughter species each fill part of the ancestral species range. Parameter

![Figure 3.—Polymorphic nucleotide sites in the Pgi intron. Symbols are as in Figure 2.](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Locus/species</th>
<th>No. of sequences</th>
<th>Total sites</th>
<th>Segregating sites</th>
<th>π</th>
<th>θ</th>
<th>( R_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ef1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. firmus</td>
<td>22</td>
<td>616</td>
<td>3</td>
<td>0.0011 (1 × 10^{-7})</td>
<td>0.0013 (7 × 10^{-7})</td>
<td>0</td>
</tr>
<tr>
<td>G. pennsylvanicus</td>
<td>14</td>
<td>616</td>
<td>4</td>
<td>0.0018 (2 × 10^{-7})</td>
<td>0.0020 (1.5 × 10^{-6})</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cam</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. firmus</td>
<td>24</td>
<td>311</td>
<td>3</td>
<td>0.0025 (2 × 10^{-7})</td>
<td>0.0026 (2.7 × 10^{-6})</td>
<td>1</td>
</tr>
<tr>
<td>G. pennsylvanicus</td>
<td>16</td>
<td>309</td>
<td>2</td>
<td>0.0012 (3 × 10^{-7})</td>
<td>0.0020 (2.1 × 10^{-6})</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cyt-c</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. firmus</td>
<td>22</td>
<td>298</td>
<td>16</td>
<td>0.0097 (1.6 × 10^{-6})</td>
<td>0.0147 (3.5 × 10^{-5})</td>
<td>4</td>
</tr>
<tr>
<td>G. pennsylvanicus</td>
<td>16</td>
<td>297</td>
<td>8</td>
<td>0.0067 (1.7 × 10^{-6})</td>
<td>0.0081 (1.3 × 10^{-5})</td>
<td>2</td>
</tr>
<tr>
<td><strong>Pgi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. firmus</td>
<td>19</td>
<td>746</td>
<td>37</td>
<td>0.0127 (2.8 × 10^{-6})</td>
<td>0.0142 (3 × 10^{-5})</td>
<td>10</td>
</tr>
<tr>
<td>G. pennsylvanicus</td>
<td>14</td>
<td>749</td>
<td>38</td>
<td>0.0162 (1.7 × 10^{-6})</td>
<td>0.0160 (4 × 10^{-6})</td>
<td>7</td>
</tr>
</tbody>
</table>

Values of θ and π are given per site, with variance of estimates in parentheses; \( R_m \) is the minimum number of recombination events per locus based on the presence of possible recombination products.
TABLE 3
Polymorphism and divergence between species of Gryllus

<table>
<thead>
<tr>
<th>Locus/species comparison</th>
<th>Avg. no. differences</th>
<th>Fixed diff.</th>
<th>Polym. 1 monom. 2</th>
<th>Polym. 2 monom. 1</th>
<th>Shared polym.</th>
<th>Polym./divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ef1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fir-pen</td>
<td>0.903</td>
<td></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0.867</td>
</tr>
<tr>
<td>fir-ovi</td>
<td>0.864</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.929</td>
</tr>
<tr>
<td>fir-vel</td>
<td>3.0</td>
<td></td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0.265</td>
</tr>
<tr>
<td>pen-ovi</td>
<td>1.143</td>
<td></td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1.053</td>
</tr>
<tr>
<td>pen-vel</td>
<td>3.5</td>
<td></td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0.351</td>
</tr>
<tr>
<td>ovi-vel</td>
<td>3.5</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cam</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fir-pen</td>
<td>0.844</td>
<td></td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0.963</td>
</tr>
<tr>
<td>fir-ovi</td>
<td>4.0</td>
<td></td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.202</td>
</tr>
<tr>
<td>fir-vel</td>
<td>6.50</td>
<td></td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0.124</td>
</tr>
<tr>
<td>pen-ovi</td>
<td>4.438</td>
<td></td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0.139</td>
</tr>
<tr>
<td>pen-vel</td>
<td>6.938</td>
<td></td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>0.082</td>
</tr>
<tr>
<td>ovi-vel</td>
<td>5.50</td>
<td></td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td><strong>Cyt-c</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fir-pen</td>
<td>3.830</td>
<td></td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>1.101</td>
</tr>
<tr>
<td>fir-ovi</td>
<td>5.273</td>
<td></td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>0.821</td>
</tr>
<tr>
<td>fir-vel</td>
<td>8.227</td>
<td></td>
<td>16</td>
<td>3</td>
<td>0</td>
<td>0.531</td>
</tr>
<tr>
<td>pen-ovi</td>
<td>6.938</td>
<td></td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0.343</td>
</tr>
<tr>
<td>pen-vel</td>
<td>7.563</td>
<td></td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>0.316</td>
</tr>
<tr>
<td>ovi-vel</td>
<td>8.50</td>
<td></td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>PGI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fir-pen</td>
<td>11.613</td>
<td></td>
<td>12</td>
<td>13</td>
<td>25</td>
<td>0.910</td>
</tr>
<tr>
<td>fir-ovi</td>
<td>17.053</td>
<td></td>
<td>34</td>
<td>3</td>
<td>2</td>
<td>0.620</td>
</tr>
<tr>
<td>fir-vel</td>
<td>16.526</td>
<td></td>
<td>37</td>
<td>6</td>
<td>0</td>
<td>0.640</td>
</tr>
<tr>
<td>pen-ovi</td>
<td>14.857</td>
<td></td>
<td>35</td>
<td>3</td>
<td>2</td>
<td>0.808</td>
</tr>
<tr>
<td>pen-vel</td>
<td>15.857</td>
<td></td>
<td>38</td>
<td>6</td>
<td>0</td>
<td>0.755</td>
</tr>
<tr>
<td>ovi-vel</td>
<td>17.00</td>
<td></td>
<td>38</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

The first column gives three-letter abbreviations for species compared. $k$ is the average number of differences per site. The next four columns indicate the number of fixed nucleotide differences between species, sites that are polymorphic in the first species (appearing on the left in the comparison column) but monomorphic in the second (on the right), sites that are polymorphic in the second species but monomorphic in the first, and sites that are polymorphic in both species, respectively. The right column indicates the ratio of polymorphism to divergence ($\theta/k$) where polymorphism data are from the left species in the comparison column (polymorphism was not calculated for $G. ovisopis$ and $G. veletis$ because only two sequences of each were available).

estimates were compared to expectations derived from 1000 coalescent simulations. The frequency of simulations with higher values of the WH test statistic than the observed was 0.51, so we cannot reject the model. Although $G. firmus$ and $G. pennsylvanicus$ hybridize where they come into contact, there is little evidence of introgression outside of the hybrid zone for many markers. A lack of ongoing gene flow would be consistent with the isolation speciation model. However, the estimate of the time to the common ancestor is $T = 0.05$ (in units of $2N_e$). We do not have good estimates for $N_e$ in crickets, but if $N_e$ is $10^4$–$10^5$, the $T$ would be only $10^4$–$10^5$ generations. Thus, if effective gene flow is absent, the high levels of shared polymorphism would indicate a very recent origin of these two cricket species. Some shared polymorphism and low sequence divergence suggest that the common ancestor of $G. firmus$, $G. pennsylvanicus$, and $G. ovisopis$ also existed fairly recently.

Tests of natural selection: HKA tests compare levels of polymorphism within a species and divergence between that species and a close relative. We examined polymorphism to divergence ratios for all six possible locus pairs where they come into contact, there is little evidence of introgression outside of the hybrid zone for many markers. A lack of ongoing gene flow would be consistent with the isolation speciation model. However, the estimate of the time to the common ancestor is $T = 0.05$ (in units of $2N_e$). We do not have good estimates were similar across loci. However, in comparisons of each of these species with $G. ovisopis$ and the more distantly related $G. veletis$, polymorphism/divergence values were generally low for $Cam$ and high for $PGI$. While the $Cam$-$PGI$ comparisons yielded HKA test $P$ values greater than but close to 0.05, none of the tests indicated
Figure 4.—Neighbor-joining trees using Jukes-Cantor distances of individual alleles at each of four loci. To emphasize the distribution of alleles among species, *G. pennsylvanicus* alleles are indicated by dark background with white lettering; *G. ovisopis* alleles are indicated by light gray background.
Figure 4.—Continued.
statistical departure from neutrality in comparisons between individual pairs of loci or when all loci were combined in a single test. Because G. firmus and G. pennsylvanicus exhibited no fixed differences and many shared haplotypes, we combined these two species into a single taxon for additional HKA tests vs. G. veletis. In this case, the Pgi vs. Cam comparison did indicate departure from neutrality \( (P = 0.040) \). G. firmus and G. pennsylvanicus differ little with respect to allele frequencies yet they are clearly not a single equilibrium population, so the statistical validity of combining them for this test is not clear. However, it does highlight the fact that the relationship between polymorphism and divergence differs substantially between Pgi and Cam.

The ratio of polymorphism to divergence for the Pgi intron is also significantly higher than the corresponding ratio for mitochondrial coding sequences (1621 bp of COI and COII, Willett et al. 1997). HKA tests (accounting for reduced \( N_e \) in mtDNA) comparing G. firmus and G. pennsylvanicus with G. veletis were both significant \( (P = 0.016 \) and \( P = 0.020 \), respectively). On the basis of comparisons with mtDNA sequences, Pgi exons have previously been shown to exhibit evidence of balancing selection (Katz and Harrison 1997). Because it appears that the intron has been subject to recombination, we might expect a portion proximal to the selected site(s) to show a more extreme relationship between polymorphism and divergence. Hence, there may be heterogeneity in the ratio of polymorphic sites to fixed differences along the sequence (McDonald 1998). However, we found no significant heterogeneity in the Pgi intron (Kolmogorov-Smirnov test statistic 0.04, \( P = 0.22 \)). Thus we could not identify specific variants in the intron that might be linked to presumptive selected sites in the coding region.

**DISCUSSION**

**Gene genealogies and diversification within the genus Gryllus:** Speciation involves the splitting of a single interbreeding population into two differentiated populations between which there are intrinsic barriers to gene exchange (Harrison 1998). Observed patterns of variation in the daughter populations reflect the operation of evolutionary forces over recent history and potentially provide insights into how speciation occurred. The amounts of polymorphism and the structure of gene genealogies will depend on the timing of speciation events, historical population sizes, the impact of natural selection and recombination, and the extent of hybridization and introgression. For the closely related field crickets examined in this study, levels of intron sequence variation differ substantially among loci, much of the polymorphism appears to predate divergence of two or even three of the species, and many shared alleles (or groups of similar alleles) show little or no geographical structure.

That ancestral polymorphism has persisted through the divergence of G. firmus, G. pennsylvanicus, and G. aestivalis is consistent with the notion that speciation has occurred rapidly, that \( N_e \) has remained large, and that a small number of genes are responsible for divergence and reproductive isolation in these crickets. If alternate fixation of alleles at relatively few loci is sufficient for reproductive isolation to occur, then at most loci polymorphisms may persist for substantial lengths of time after interbreeding ceases (Hilton et al. 1994; Ting et al. 2000; Wu 2001). Even substantial gene flow might not affect species integrity so long as there is no introgression of “speciation genes.” Only after substantial time has passed from the speciation event \([\text{typically} > 2N_e \text{ generations (Maddison 1997)}]\) will taxa approach reciprocal monophyly for variation at most loci (Tajima 1983; Neigel and Avise 1986; Harrison 1991; Hey 1994).

An alternative explanation for shared haplotypes is that gene flow subsequent to initial divergence has erased patterns of exclusivity for some loci. If ongoing interspecific gene flow were occurring we would expect greater similarity among alleles from geographically close populations. That is, under a stepping-stone population model (which is appropriate for taxa of low vagility such as crickets), local demes should be more similar to those with which they are directly exchanging genes than to more distant demes with which they would have little or no contact. Thus we would expect, for example, that northern G. firmus samples would be more similar to northern G. pennsylvanicus samples than to more distant G. firmus due to isolation by distance. Consequently, in this case with no correlation between genetic distance and geographic proximity, polymorphism maintained from the common ancestor is the most plausible explanation for the observed deep coalescence of nuclear loci. Although our results suggest that divergence \( (\text{cessation of gene flow}) \) between the species occurred recently, we cannot readily determine whether this was the initial divergence event or the cessation of gene flow between populations that had diverged previously.

The estimated time since divergence between G. firmus and G. pennsylvanicus \( (0.05 \times 2N_e \text{ generations}) \) suggests that branch lengths are substantially shorter than those that would be necessary for drift to eliminate shared variation \( (i.e., 2N_e \text{ generations}) \). Furthermore, Palumbi et al. (2001) have suggested that there is a relatively high probability of exclusivity for nuclear genes only if the length of the branch leading to an exclusive mtDNA clade is more than three times the diversity within the clade. The ratio of branch length diversity for mitochondrial sequences in G. firmus and G. pennsylvanicus ranges from \( \sim 0.5 \) to \( 2 \) (mtDNA data from Willett et al. 1997) and thus does not satisfy the “three times rule.” Therefore, polyphyly for nuclear loci is not unexpected, given the mtDNA tree.

Of course, genetic differences must underlie observed
morphological, behavioral, ecological, and reproductive differences between the closely related cricket species. Previous surveys of genetic variation across the hybrid zone between *G. firmus* and *G. pennsylvaniaicus* have revealed evidence of such differentiation, but the overall impression from multiple surveys is that these two species have diverged very little. Allozyme surveys (25 loci) of species in the genus Gryllus revealed no fixed differences between *G. firmus* and *G. pennsylvaniaicus*, with a Nei’s D (Nei 1972) of only 0.02–0.03 (Harrison 1979, 1986). Three loci (Esterase, Peptidase-I, and Peptidase-3) showed significant allele frequency differences. The hybridizing cricket species can be distinguished on the basis of mtDNA haplotype (Harrison et al. 1987; Willett et al. 1997), but as discussed above, neither of the species appears to be an exclusive group, although regional groups of populations within species are exclusive for mtDNA. Finally, previous efforts to characterize nuclear gene markers involved RFLP analysis of anonymous nuclear regions. Of 52 pUC clones used as hybridization probes to digests of cricket genomic DNA, many appeared to exhibit evidence of shared polymorphisms (for presence/absence of restriction sites) and only three uncovered fixed or nearly fixed differences (Harrison and Bogdanowicz 1997). Taken together, these results support the impression derived from the intron sequence data that *G. firmus* and *G. pennsylvaniaicus* have diverged very little and that for much of the genome, ancestral polymorphisms are still segregating in the cricket populations. Thus, evidence from Gryllus is consistent with the theoretical expectation that a large portion of the genome frequently remains common to closely related species well after speciation has occurred.

Patterns of polymorphism and divergence in several groups of Drosophila are very similar to those seen in the field crickets. Many nuclear loci appear to be paraphyletic or polyphyletic with respect to one or more recognized species in the *Drosophila melanogaster* species group (Hey and Kliman 1994; Kliman et al. 2000), *D. pseudoobscura* and close relatives (Wang et al. 1997; Machado et al. 2002), and the *D. virilis* species group (Hilton and Hey 1996). However, in contrast to patterns seen for other loci, variation at a putative speciation gene (*OldH*) has been shown to resolve *D. simulans* and its close relatives *D. sechellia* and *D. mauritiana* as monophyletic species (Ting et al. 2000). The expectation is that such regions are also present in the Gryllus genome and mark sites of recent lineage-specific selective sweeps and/or loci that contribute to reproductive isolation.

**Patterns of variation and the role of natural selection:**

Levels of polymorphism varied 10-fold among the four loci we sampled. However, on average, amounts of variation in Gryllus appear to be comparable to those found in Drosophila. Moriyama and Powell (1996) summarized available information from *D. melanogaster*, *D. simulans*, and *D. pseudoobscura*. Noncoding autosomal regions from *D. melanogaster* exhibited π values between 0.0005 and 0.0265 with a mean of 0.0117. Although fewer noncoding regions were available, diversity was somewhat higher in *D. simulans* (mean = 0.0189, range = 0.0058–0.0338) and *D. pseudoobscura* (mean = 0.0170, range = 0.0100–0.0209). Two of the four cricket intron sequences (*Pgi* and *Cyt-c*) fall within observed ranges of diversity values for Drosophila, while the other two cricket loci are less variable.

Population sizes and rates of diversification are characteristics of lineages and therefore cannot explain differences in patterns of variation among loci within a lineage. To explain locus- or genomic region-specific properties, we must invoke differences in mutation rates among gene regions or differences in the history of selection. The neutral theory predicts that if population size is constant, the number of alleles present at any given time will depend on the mutation rate, but that the time to coalescence will not. Variation among loci in θ (4Nμ) within a species may indicate variation in the mutation rate if population size is assumed to be similar for all autosomal loci. Population subdivision can elevate effective population size (Wright 1943) and can stretch out coalescence times (Wakeley 2000). This would violate the assumption of panmixia made by the isolation species model and could be an additional explanation of the high degree of shared polymorphism. However, we found no evidence of population subdivision for these loci.

Natural selection also affects levels of polymorphism. Balancing selection can maintain ancestral polymorphism, causing deep coalescence. In contrast, directional selection will cause reduced variation through selective sweeps. If mutation rates are equal for different genomic regions, the depth of coalescence will vary depending on the type and intensity of selection, the rate of recombination, and the proximity of the site(s) under selection to the gene region used in genealogical analysis.

One way to distinguish selection from mutation rate is with the HKA test. Neutral theory predicts that polymorphism will be proportional to divergence regardless of mutation rate. Hence significant differences in such ratios are indicative of selection. On the basis of early allozyme studies (Harrison 1977) and subsequent characterization of coding sequences for *Pgi* in *G. veletis* and *G. pennsylvaniaicus* (Katz and Harrison 1997), we started with the expectation that the *Pgi* locus might provide evidence for balancing selection. For most comparisons between intron sequences, *Pgi* has the highest ratio of polymorphism to divergence, which can be explained by a history of balancing selection. Comparisons with mtDNA also indicate a significantly greater polymorphism/divergence ratio, again consistent with the balancing selection scenario. Presumably *Pgi* and *Cam* represent ends of a continuum with respect to levels of
variability, and these loci may well have been subject to opposing selective forces.

Our failure to find compelling evidence for selection on Pgi is perhaps not entirely surprising. In other organisms, balancing selection has been difficult to demonstrate (Kreitman and Akashi 1995; Hey 1999). Departures from neutrality consistent with balancing selection are evident from patterns of variation for several Drosophila genes (e.g., Aguadé et al. 1992; Kirby and Stephan 1995; Oggers et al. 1995; Eanes et al. 1996; Wayne et al. 1996), but the power of available statistical tests for selection is generally weak (Simonsen et al. 1995). Moreover, alternative tests (e.g., HKA, Tajima’s D, and McDonald-Kreitman) often yield conflicting results for a particular locus (see Moriyama and Powell 1996). So while it is likely that many loci have been subject to balancing selection, Adh remains one of the few clear examples (Hudson et al. 1987).

Pgi has been examined for evidence of selection in Drosophila. HKA tests involving the noncoding region of Pgi in Drosophila yield significant differences from a few other loci and the difference from Adh is highly significant (<0.005; Moriyama and Powell 1996). However, in Drosophila the differences are due to a lack of polymorphism at Pgi, which is one of the least variable loci in both D. melanogaster (π = 0.0018) and D. simulans (π = 0.0058). Given the extreme functional conservation of the glycolytic pathway, Pgi almost certainly performs the same function in both taxa. Thus, the striking difference in levels of polymorphism between crickets and flies may reflect different selective regimes.

An alternative to balancing selection is that the elevated polymorphism level at Pgi is related to a higher recombination rate and the lower polymorphism observed for other loci could be due to lower recombination rates in combination with selective sweeps. Under this scenario, polymorphism at Pgi could be neutral but a greater recombination rate allows it to escape the effects of selective sweeps. We cannot discount this hypothesis entirely, although in our view the bulk of the evidence points to balancing selection.

Conclusions: Given sufficient time between divergence events, recognized species or populations will become monophyletic or exclusive groups. However, when populations have diverged recently or when divergence events have occurred in rapid succession, patterns of exclusivity are not expected, particularly for nuclear loci. While often considered to be an annoyance from a purely phylogenetic perspective, this phenomenon allows for important inferences about very recent evolutionary events. In this case, genealogical analysis suggests that three species of field crickets diverged recently, that most of these species’ genomes have diverged little from their common ancestor, and that relatively few genes have been involved directly in the speciation process. In addition, extensive polymorphism and deep coalescence at one locus (Pgi) may reflect maintenance by balancing selection, while low polymorphism characteristic of a selective sweep is evident at a second locus (Cam), in spite of the fact that the limited variation appears to predate the G. firmus-G. pennsylvaniae speciation event. Thus, nuclear gene genealogies may have limited utility in resolving closely related taxa, yet they can provide important insights into the historical, demographic, and selective forces that shape speciation and ultimately give rise to broader phylogenetic patterns.

We thank S. Bogdanowicz for technical assistance, members of the Harrison lab for helpful discussions, and J. Feder and an anonymous referee for thoughtful reviews of an earlier version of the manuscript. This work was supported by a National Science Foundation (NSF)/Alfred P. Sloan Foundation postdoctoral fellowship in molecular evolution to R.E.B. and by NSF population biology grant DEB-981530 to R.G.H.

LITERATURE CITED


Communicating editor: J. Hey