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Sorted gene genealogies and species-specific nonsynonymous substitutions point to putative postmating prezygotic isolation genes

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Abstract

Not all genes contribute equally to reproductive isolation. In the *Allonemobius socius* complex of crickets, reproductive isolation is primarily accomplished via postmating prezygotic barriers. We show that two ejaculate protein-coding genes exhibit patterns of evolution consistent with a putative role as speciation genes. Both genes express male ejaculate proteins transferred to females during copulation and were previously identified through comparative proteomics. We found gene genealogies indicating advanced degrees of lineage sorting, and fixed nonsynonymous substitutions and elevated ω values on the mutational steps separating species, between both pairs of species, on the haplotype networks of these genes compared to other candidate and control genes. At a contact zone between two members of the species complex, these genes maintained species-specificity of alleles despite ongoing gene flow. The putative speciation genes *arginine kinase* (AK) and *apolipoprotein A-1 binding protein* (APBP) are two of the first examples of sperm maturation, capacitation, and motility related proteins that show evidence of fixed nonsynonymous substitutions between species-specific alleles that may lead to reproductive isolation. Our results show that when speciation is ongoing and insufficient time has passed for nucleotide variation to accumulate, hypothesis testing based on haplotype networks and gene trees are more powerful than sequence-based population genetic metrics at detecting signatures of positive selection that may have led to speciation.

Introduction

~~Not all genes contribute equally to reproductive isolation during speciation.~~ ‘Speciation’ (Wu, 2001; Wu & Ting, 2004; Nosil & Schluter, 2011), ‘isolation’ (Rieseberg, Church & Morjan, 2004), or ‘barrier’ (Noor & Feder, 2006) genes are expected to show very different patterns of evolution compared to genes that are not directly involved in reproductive isolation when species are still undergoing lineage sorting (Wu 2001). Therefore we expect to find putative speciation genes among those genes that become fixed for alternative alleles within each incipient species early in the process of divergence, with said alleles rarely crossing the species boundary in sympatry (Ting, Tsaur & Wu, 2000; Dopman et al., 2005).

Rapidly evolving reproductive proteins that can affect fertilization success have an important role in the evolution of postmating prezygotic reproductive isolation. Many reproductive genes are known to evolve rapidly in a variety of organisms (Civetta & Singh, 1998; Swanson & Vacquier, 2002; Clark, Aagaard & Swanson, 2006; Panhuis & Swanson, 2006; Snook et al., 2009). In *Drosophila* where some of the most extensive work has been done, genes that show male-biased expression evolve faster compared to female-biased and somatically expressed genes (Zhang, Hambuch & Parsch, 2004; Zhang & Parsch, 2005; Metta et al., 2006; Pröschel, Zhang & Parsch, 2006; Haerty et al., 2007), and seminal fluid proteins in particular tend to show an excess of nonsynonymous substitutions (Begun et al., 2000; Swanson et al., 2001; Wagstaff & Begun, 2005; Almeida & DeSalle, 2008). Similar patterns have also been observed in mice and primates (Clark & Swanson, 2005; Karn et al., 2008; Ramm et al., 2008; Turner, Chuong & Hoekstra, 2008; Dean et al., 2009). Using a proteomics approach on insect spermatophores to isolate male reproductive protein coding-genes that can directly interact with female counterparts has proved to be an efficient way of narrowing prospects in the search for putative speciation genes (Andrés, Maroja & Harrison, 2008; Marshall et al., 2011).

The male ejaculate proteome comprises sperm-expressed proteins and seminal fluid proteins. Sperm not only contribute half of the diploid genome, but are also involved in sperm-egg interactions including egg activation and deliver paternal factors during fertilization (Dorus et al., 2006). Seminal fluid proteins, the majority of which are produced by male accessory glands, contain conserved functional classes of peptides and pro-hormones that are involved in sperm binding, proteolysis, lipid metabolism, and immune function (Mueller et al., 2004; Chapman & Davies, 2004; Poiani, 2006; Avila et al., 2011). Once transferred into the female reproductive tract, these proteins can initiate a wide-range of physiological functions including increased egg production and oviposition, decreased receptivity, decreased lifespan, and increased feeding in females (reviewed in (Avila et al., 2011). The interacting female counterparts to these ejaculate proteins (EPs) are not well known (Ram, Ji & Wolfner, 2005; Ram & Wolfner, 2007; Snook et al., 2009) though genomic data is proving to be invaluable for identifying candidates (Findlay et al., 2014). The evolution of EPs has been hypothesized to be driven by one or more processes including female sperm preference, sperm competition, and sexual conflict (Mueller et al., 2004; Snook et al., 2009). Here, we show through multiple lines of evidence that two EP-coding genes in the *Allonemobius socius* complex of crickets show patterns of molecular evolution and gene genealogies consistent with a putative role as speciation genes.

The *A. socius* complex of ground crickets, *A. socius*, *A. fasciatus*, and *A. sp. nov.* Tex, represents a powerful system to explore the hypothesized link between EP divergence and reproductive isolation. Members of this complex are primarily isolated from one another by two postmating, prezygotic phenotypes – conspecific sperm precedence (Gregory & Howard, 1994; Howard et al., 1998a,b; Marshall, 2004) and the superior ability of conspecific males to induce females to lay eggs (Gregory & Howard, 1993; Howard et al., 1998b). Two other compelling features of this organismal system are species boundaries that remain intact in sympatry despite some gene flow (Howard, 1986; Howard & Waring, 1991; Traylor et al., 2008) and the very recent nature of divergence between these species (i.e., within the last 30,000 years; (Marshall, 2004, 2007). Indeed, divergence is so recent that few species-specific alleles have been identified; for example, only 2 of 17 allozyme markers (Howard, 1983), 2 of 5,400 AFLP markers (Howard et al., 2002), ~21 of 1,660 thorax proteins and ~33 of 922 ejaculate proteins (Marshall et al., 2011) and 1 of 16 randomly chosen reproductive genes spanning >7,500 bp of coding sequence (Marshall et al., *unpublished data*), yield evidence of species specificity. Taken together, the above data suggest that while there is sufficient genetic divergence to produce reproductive isolation and maintain species boundaries in sympatry, the vast majority of genes show no evidence of divergence and thus, no lineage sorting. In all, the *A. socius* complex represents a system whereby speciation is ongoing with relatively few genes contributing to the postmating, prezygotic reproductive isolation between species. Therefore, if we can identify those ejaculate and female reproductive tract genes that exhibit signatures of positive selection, and maintain species-specificity in sympatry, we will gain insight into the genes that contribute to reproductive isolation and ultimately are involved in driving speciation.

In this study, we expanded analyses from a previous study comparing EPs between the species *A. socius* and *A. fasciatus* (Marshall et al., 2011) by including more genes and an additional species, *A. sp. nov.* Tex (Traylor et al., 2008). Specifically, longer fragments of the five original proteins (ACG69, AK, APBP, EJAC-SP, SPI) plus two additional EPs (GOT, SPAG6) were compared for patterns of nucleotide variation, evidence of lineage-specific positive selection and different degrees of lineage sorting, and species-specificity of alleles in the contact zone between *A. socius* and *A. fasciatus*. These combined analyses point toward an important role for some but not all examined EPs during the evolution of reproductive isolation within this complex of crickets.

Methods

Background

Striped ground crickets of the *A. socius* complex inhabit moist grasslands across North America and do not show significant habitat isolation (Howard 1986). The three species *A. socius*, *A. fasciatus*, and *A. sp. nov.* Tex form two contact zones, one between *A. fasciatus* (north) and *A. socius* (south) from Illinois to New Jersey (Howard & Waring, 1991), and one between *A. sp. nov.* Tex (west) and *A. socius* (east) near the Louisiana – Texas state line (Traylor et al., 2008). *A. fasciatus* and *A. socius* seem to have diverged from a common ancestor approximately 30,000 years ago, and *A. sp. nov.* Tex seems to have subsequently diverged from *A. socius* approximately 24,000 years ago (Marshall, 2004, 2007). They have previously been shown to be isolated primarily via postmating prezygotic reproductive isolation (Howard et al., 2002; Marshall, 2004; Marshall & DiRienzo, 2012).

Population and gene sampling

Crickets were collected from each population and genotyped in the lab via allozymes (Isocytrate dehydrogenase and Hexokinase) to determine species identity (Howard, 1983, 1986). Sampling localities spanned the range of each species. *A. socius* populations were sampled near

Texarkana, AR (AR), Bottom, NC (Bot), Mt. Vernon, IL (IL), Pleasantville, NJ (Mi), Ruston, LA (LA), Gastonia, NC (NC), and Ardmore, OK (OK). *A. fasciatus* populations were sampled near Akron, OH (Akn), Frankfort, IL (FF), and New Paltz, NY (NP). *A. sp. nov.* Tex populations were sampled near Terrell, TX (Tx20), Royse City, TX (Tx30), and Gainesville, TX (Tx35). Contact zone populations of *A. fasciatus* and *A. socius* were sampled from two habitats at a single location in Kenna, WV. *A. fasciatus* was collected from a hillside habitat, which we call Kenna Hill (KH), and *A. socius* was collected along the base of hill near a creek which we call Kenna Creek (KC). We did not have samples from the contact zone between *A. socius* and *A. sp. nov.* Tex. General maintenance protocols followed Marshall et al (2009).

We dissected male accessory glands and testes from three individuals per allopatric population and 9 individuals per contact zone population. cDNA was synthesized from each tissue using RNA isolated via an Ambion RNAqueous-4PCR (#AM1914) kit and standard protocols for 1st strand cDNA synthesis. General PCR and sequencing procedures followed Marshall et al (2011). Standard PCR chemistry was followed with annealing temperatures between 50-60 °C depending on individual primer melting temperatures (primers used are shown in Supplementary Table 1). We compared nucleotide sequences of five candidate EP genes with two control EP genes. ~~Among~~ the five candidate genes, two were chosen based on species-specific proteome profiles (Marshall et al., 2011): 1) *arginine kinase* (AK), a phosphotransferase enzyme expressed in the sperm that may be involved in sperm motility, capacitation or the acrosome reaction (Strong & Ellington, 1993; Niksirat et al., 2015); 2) *apolipoprotein A-1 binding protein* (APBP), a phosphoprotein expressed in sperm and hypothesized to be involved in sperm capacitation (Jha et al., 2008). Two were chosen based on previous sequencing data showing species-specific molecular variation: 3) *ejaculate serine protease* (EJAC-SP), an abundant accessory gland-expressed serine protease previously shown to be involved in the induction of egg laying in successfully mated females (Marshall et al., 2009); 4) *aspartate aminotransferase* (GOT), a pyridoxal-phospate-dependent aminotransferase expressed in the testis and an allozyme historically used to diagnose species identity among *A. socius* complex crickets (Howard, 1983, 1986). The last candidate gene was chosen based on a review of sperm biology literature: 5) *sperm-associated antigen 6* (SPAG6), important for sperm flagellar motility and the structural integrity of the central apparatus (Neilson et al., 1999; Sapiro et al., 2002).

The control genes had non species-specific proteome profiles (Marshall et al., 2011) and were: 6) *serpine inhibitor* (SPI), a testis-expressed serine-type endopeptidase inhibitor; 7) *acg69* (ACG69), a protein of unknown function expressed in the accessory glands. Sequences formatted as haplotypes are available from NCBI GenBank PopSets 372477483 (AK), 372477513 (APBP), 372477527 (EJAC-SP), 372477535 (GOT), 372477555 (SPAG6), 372477561 (SPI), 372477571 (ACG69).

Sequence evolution-based analyses

Male biased genes have been shown to exhibit patterns of molecular evolution associated with relaxed selective constraints or strong positive selection, such as higher rates of nonsynonymous substitutions (Zhang, Hambuch & Parsch, 2004). We investigated multiple metrics of molecular sequence evolution to test for evidence of selection and a departure from neutral sequence evolution. We applied Tajima's D and Fu and Li's D tests to each gene to look for evidence of departure from neutral allelic distributions within species (Tajima, 1989; Fu & Li, 1993). We compared polymorphism within species to divergence between species using HKA tests (Hudson, Kreitman & Aguadé, 1987), and tested for differences in these ratios at each branching node of the species tree.

Next, we compared polymorphism and divergence between synonymous and nonsynonymous sites within each gene at each branching node of the species tree. We compared ω, the rate ratios of synonymous substitutions per synonymous site κ_a (d_N) and nonsynonymous substitutions per nonsynonymous site κ_s (d_S). We used McDonald-Kreitman tests to compare the ratio of nonsynonymous to synonymous intraspecific polymorphisms to the ratio of nonsynymous to synonymous fixed differences between species (McDonald, Kreitman & others, 1991). All tests were based on sequences aligned in BioEdit v.7.0.5.3 (Hall, 1999) and metrics calculated using DnaSP v.5.10.01 (Librado & Rozas, 2009). For HKA tests, we used the program hka provided by Jody Hey (Wang & Hey, 1996).

Gene genealogy-based analyses

Evolutionary relationships between species are tested with phylogenetic trees while hypotheses of intraspecific relationships benefit from haplotype network-based approaches (Posada & Crandall, 2001). Because our species are recently diverged, we used both tree-based and haplotype network-based analyses to detect interesting patterns of gene evolution.

We used statistical parsimony haplotype networks (Templeton, Crandall & Sing, 1992) of alleles from all three species to test for species-specificity of alleles. We used TCS (Clement, Posada & Crandall, 2000) to generate the haplotype networks using only allopatric individuals. Species-specific alleles were defined as those found only within each respective species. Common or shared alleles were those observed in more than one species. Once alleles were designated common or specific to a species, we turned to the *fasciatus* - *socius* contact zone. We looked at nine individuals each of contact zone *A. fasciatus* and *A. socius* and determined what types of allele these contact zone individuals possessed. As noted above, these individuals had previously been designated as fully (homozygous) *A. fasciatus* or *A. socius* based on allozymes. We used Fisher's exact tests with Freeman-Halton extensions for 2x3 contingency tables to determine the probability of observing the distribution of *fas* vs. *soc* vs. shared alleles for each gene.

We tested for lineage-specific positive selection on individual gene tree topologies using the Genetic Algorithm (GA) Branch method (Pond & Frost, 2005) via the Datamonkey webserver of the HyPhy package (Delport et al., 2010). GA Branch uses a genetic algorithm that allows estimates of the nonsynonymous to synonymous substitution rate ratio ($d_N/d_S = \kappa_a/\kappa_s = \omega$) to vary freely across branches within a phylogeny and compares models with different ω classes. Only allopatric individuals were included in the analysis and neighbor-joining trees used by GA Branch were generated natively within Datamonkey.

The genealogical sorting index (gsi) reflects the degree of lineage sorting of individual gene genealogies that occurs during speciation, with values ranging from zero (complete polyphyly) to 1 (complete monophyly) (Cummings, Neel & Shaw, 2008). We calculated gsi for each gene using the online server (www.genealogicalsorting.org) with gene trees including both allopatric and contact zone individuals. Sequences were phased in DnaSP prior to tree building for all genes except APBP, which had no heterozygous individuals. Sorting is more difficult to observe in phased data. We generated maximum likelihood gene trees with PhyML 3.0 (Guindon et al., 2010) via the Mobyle server (Neron et al., 2009). We used nearest neighbor interchange (NNI) tree search and HKY85 as our nucleotide substitution model. MEGA6 (Tamura et al., 2013) was used to visualize these trees.

Results

Sequence evolution-based analyses

We found a general lack of both synonymous and nonsynonymous nucleotide variation among all EP genes we investigated (Table 1). The Watterson estimator $\theta=4N_e\mu$ ranged from 0.001 to 0.011. Levels of θ in the EP candidate genes were approximately an order of magnitude lower than the control genes, although this difference was not statistically significant (*fas* - Mann-Whitney $U = 0$, $P = 0.051$; *soc* - Mann-Whitney $U = 4$, $P = 0.688$; Tex - Mann-Whitney $U = 4.5$, $P = 0.845$). In no cases were Tajima's D or Fu and Li's D tests significantly different from neutral expectations (Table 1) (all $P > 0.1$).

To compare polymorphism within species to divergence between species, we used a standard multilocus HKA test and HKA outlier tests for each branching event. We included all loci and performed 9999 rounds of coalescent simulations. The multilocus HKA test did not find a significant departure from neutral expectations for the first branching event between *A. fasciatus* and the two other species (X^2 $P = 0.916$). The outlier cell, which was *A. fasciatus* for polymorphism in ACG69, was not significantly different in its pattern of polymorphism to divergence ($P = 0.68$). The multilocus HKA test did find a significant departure from neutral expectations for the second branching event between *A. socius* and *A. sp. nov.* Tex. (X^2 $P = 0.012$). However, the outlier cell, which was polymorphism at GOT in *A. sp. nov.* Tex., was not significantly different in its pattern of polymorphism to divergence ($P = 0.06$).

We compared the rate ratios of nonsynonymous to synonymous substitutions $\omega = \kappa_a/\kappa_s$ at each branching event of the species tree. When ω is larger than 1 and the nonsynonymous substitution rate exceeds the synonymous substitution rate, positive or diversifying selection is inferred. When ω is smaller than 1, negative or purifying selection is inferred. However, ω = 1 is recognized as a conservative threshold because the average κ_a is expected to be much smaller than κ_s given the expectation of widespread purifying selection acting on functional genes (Nielsen, 2001). Therefore the value ω = 0.5 has been suggested as an alternate cutoff for the detection of positive selection as subsequent analyses generally indicate that such genes are indeed under positive selection (Swanson et al., 2004). In none of our genes did ω exceed 1, but in the older split between *A. fasciatus* and the other two species, ω exceeded 0.5 for the genes AK and APBP (Table 2).

We used McDonald-Kreitman tests to compare the ratio of nonsynonymous to synonymous intraspecific polymorphisms (P_N/P_S) to the ratio of nonsynonymous to synonymous fixed differences between species (D_N/D_S). Not all genes had fixed nonsynonymous substitutions between species and in these cases we were unable to apply the McDonald-Kreitman test. For those genes that were testable, we did not find significant differences in D_N/D_S compared to P_N/P_S at either branching event (Fisher's exact test $P = 0.07 \sim 1$) (Table 2).

Gene genealogy-based analyses

The statistical parsimony haplotype networks generated using allopatric individuals of all three species showed the presence of only species-specific alleles in AK, APBP, and GOT, while the other genes had alleles shared between two species each (Figure 1). Within the contact zone between *A. fasciatus* and *A. socius*, AK, APBP, EJAC-SP and SPAG6 had upwards of 16 species-specific alleles out of 18 possible alleles (approximately 88%) (Figure 2). In comparison, many GOT and SPI alleles were shared between the contact zone populations. Fisher's exact tests indicated the allelic distributions were nonrandom for all genes except ACG69.

The GA Branch method detected elevated ω classes in all genes except EJAC-SP and SPAG6 (Table 3). We mapped the substitution rate changes detected by GA Branch onto the haplotype networks (Figure 1). In AK and APBP, elevated ω were detected on mutational steps between both species pairs, between *A. fasciatus* and *A. socius* and between *A. socius* and *A. sp. nov.* Tex, and were associated with one or more fixed nonsynonymous substitutions. In GOT and SPI, elevated ω were detected on mutational steps between *A. fasciatus* and *A. sp. nov.* Tex, and also within *A. sp. nov.* Tex. Elevated ω were detected on several branches in ACG69 (Figure 1).

Comparisons of genealogical sorting index values based on maximum likelihood gene trees including all sampled individuals, both allopatric and contact zone, indicated that only AK and APBP showed advanced lineage sorting for all three species (Table 4, Supplementary Figures 1 & 2). Excluding *A. sp. nov.* Tex, which had high gsi-values overall most likely due to its limited range and lack of data from its contact zone with *A. socius*, the two control genes were unsorted across the species ranges. The remaining three candidate genes showed asymmetrical lineage sorting.

Discussion

Many reproductive genes, and in particular those that are male biased, are known to evolve rapidly, often exhibiting higher rates of nonsynonymous substitutions (Zhang, Hambuch & Parsch, 2004), reduced codon usage bias (Hambuch & Parsch, 2005), and evidence suggesting they are more likely to evolve by duplication (Ellegren & Parsch, 2007). However recent divergence can hinder the application of many metrics of molecular evolution that rely on sequence variation since not enough evolutionary time has passed to allow for differences to accumulate between incipient species. Thus data from relatively recently (~30,000 years) diverged species such as ours show a general lack of both synonymous and nonsynonymous nucleotide variation among all investigated genes (Table 1 & 2). In addition, our estimates of sequence variation were also at least an order of magnitude smaller compared to other known estimates from accessory gland protein coding genes in various other species groups (Mueller et al., 2005; Wagstaff & Begun, 2005; Almeida & DeSalle, 2008), including some *Gryllus* crickets whose species are of roughly similar age (Andrés et al., 2006). Therefore, while relatively young species offer an opportunity to observe the ongoing process of the genetics of species divergence, attempting to identify putative speciation genes based on DNA sequences requires an approach that takes into account gene trees and haplotype networks, along with species trees.

The ratio of nonsynonymous to synonymous substitution rates ω is commonly used to detect signatures of selection acting upon protein coding genes (Yang & Bielawski, 2000; Nielsen, 2001, 2005; Jensen, Wong & Aquadro, 2007). The original intended application of ω was for the analysis of sequence evolution among distantly related species, though in practice, it is not uncommonly applied to sequences between closely related populations of a species (Kryazhimskiy & Plotkin, 2008). This turns out to be problematic because when sequence evolution under selection was simulated over short evolutionary timescales, representative of genetic variation segregating within a species, vs. long evolutionary timescales, intraspecific ω behaved very differently from interspecific ω (Kryazhimskiy & Plotkin, 2008). In fact, Kryazhimskiy and Plotkin show that under positive selection, ω = 1 when selection was moderate but showed asymptotic behavior and eventually decreased below 1 as the selection coefficient increased. Over short timescales, its variance also increased as θ (= 2Nμ in the paper) became smaller, making it more difficult to accurately detect positive selection.

Another complication with using ω for short evolutionary timescales is that during initial sequence divergence ω could be higher than expected because slightly deleterious nonsynonymous mutations can persist in a population for generations due to genetic hitchhiking, and a time lag before they are removed by purifying selection (Rocha et al., 2006). Based on simulations, Rocha and colleagues showed this is why unexpectedly high ω values are frequently observed among closely related (1 – 2% sequence divergence) bacteria species. As evolutionary time progresses further, they show that synonymous mutations continued to accumulate and exceeded the initial overrepresentation of nonsynonymous mutations. Therefore if ω is estimated too soon after sequence divergence, one would expect to find high rates of false positive detection.

Finally, even at longer evolutionary timescales the assumption that κ_s is effectively neutral may need reconsideration, as a survey of 16 vertebrate genomes indicates that genes with high ω are more strongly influenced by small κ_s rather than large κ_a (Wolf et al., 2009). Similar patterns are observed in *Drosophila* species, where fast evolving genes show a negative correlation between κ_a and synonymous site polymorphism π_s (Andolfatto, 2007; Macpherson et al., 2007; Jensen & Bachtrog, 2010).Thus, the interaction between linkage and selection makes it challenging to distinguish between recurrent positive selection, background selection, and Hill-Robertson effects (Hill & Robertson, 1966; Charlesworth, 1994; Andolfatto, 2007; Charlesworth et al., 2009). Therefore in order to detect adaptive evolution due to positive selection, applying combinations of metrics including ω, Tajima's or Fu and Li's *D* and site frequency spectra, as well as estimates of population size and recombination rates seems necessary (Nielsen, 2005).

We failed to detect positive selection based on ω, and estimates of *D* for all genes compared here were not significantly different from neutral expectations (Tables 1 and 2). While population bottlenecks are thus likely to have contributed to sequence variation patterns since speciation in the *A. socius* complex is thought to coincide with glaciation history (Marshall, 2004, 2007), for our data sequence evolution-based tests are generally inconclusive as to demographic reasons for why our genes might lack nucleotide variation.

Instead, tests based on individual gene genealogies and haplotype networks ~~indicated~~ AK and APBP as putative speciation genes. Within the contact zone of *A. fasciatus* and *A. socius*, AK, APBP, and EJAC-SP show significantly nonrandom patterns of allelic distributions and had no shared alleles (Figure 2). When all allopatric and contact zone individuals were examined, only the genealogies of AK and APBP indicated that these genes were more advanced in their degree of lineage sorting compared to other candidate and control genes (Table 4, Supplementary Figures 1 & 2). These patterns fit models of ongoing speciation in the face of gene flow, where speciation genes are more likely to be fixed early on during lineage divergence (Wu, 2001). Incomplete lineage sorting and introgression have been suggested to be confounding factors in understanding speciation with ongoing gene flow (Machado & Hey, 2003; Broughton & Harrison, 2003; Payseur, 2010). However, speciation genes are more likely to become fixed for species-specific alleles early in the process of speciation and therefore are expected to be relatively exempt from incomplete sorting and subject to reduced introgression. Similar patterns have been observed in *Drosophila*, field crickets, and moths (Ting, Tsaur & Wu, 2000; Dopman et al., 2005; Maroja, Andrés & Harrison, 2009; Andrés et al., 2013; Larson et al., 2013). It is possible that these genes are not the direct targets but rather linked to targets of divergent selection. Because both genes were identified through comparative proteomics (Marshall et al 2011) this seems relatively unlikely, but the genomic regions around these genes should be investigated for evidence of selective sweeps to rule out this possibility.

Many studies of reproductive proteins report evidence of positive selection acting on a subset of the genes examined, in both males (Begun et al., 2000; Swanson et al., 2001; Clark & Swanson, 2005; Wagstaff & Begun, 2005; Andrés et al., 2006; Karn et al., 2008; Ramm et al., 2008; Almeida & DeSalle, 2008; Walters & Harrison, 2010) and females (Swanson et al., 2004; Panhuis & Swanson, 2006; Lawniczak & Begun, 2007; Prokupek et al., 2008; Kelleher & Markow, 2009; Kelleher, Clark & Markow, 2011). However, there are few examples of adaptive reproductive protein evolution leading to reproductive isolation outside of gamete recognition proteins (e.g. (Geyer & Palumbi, 2003; McCartney & Lessios, 2004; Springer & Crespi, 2007). Our putative speciation genes AK and APBP two of the first examples of sperm maturation and capacitation related proteins that show evidence of fixed nonsynonymous substitutions ~~between~~ species-specific alleles ~~leading to~~ reproductive isolation. In contrast to the other genes examined, fixed nonsynonymous substitutions and elevated ω values only on the mutational steps separating species on the haplotype network of APBP, and to a less exclusive extent in AK and GOT, indicate that these EPs may have evolved under positive selection and contribute to the reproductive isolation between these species (Table 3, Figure 1). We had previously observed this pattern between *A. fasciatus* and *A. socius* for both AK and APBP (Marshall et al 2011), but finding the same pattern in the mutational steps between *A. socius* and *A. sp. nov.* Tex with different species-specific nonsynonymous substitutions emphasizes the potential importance of these candidates.

Whether there are functional consequences to the species-specific nonsynonymous substitutions in AK and APBP needs to be investigated further. Since both candidates were identified by proteomic screens, we hypothesize that an interaction between each male EP and the female reproductive tract during capacitation is responsible for the postmating prezygotic isolation observed between the *A. socius* complex species. AK is a phosphagen kinase that catalyzes ATP-regeneration and energy transport in invertebrates and some protozoa (Ellington, 2001; Noguchi, Sawada & Akazawa, 2001; Uda et al., 2006). Insects and other ecdysozoans possess AK as their sole phosphagen system for cellular energy metabolism, and accordingly, arginine phosphate and its phosphagen kinase AK are found primarily in muscles, but also in sperm and compound eyes (Strong & Ellington, 1993; Kucharski & Maleszka, 1998; Ellington, 2001). The possible roles of AK as an EP can be related to sperm motility (Strong and Ellington 1993), capacitation, or the acrosome reaction (Niksirat et al., 2015). Two structural loops and several active sites near them are the proposed interaction interface of AK with the guanidinium groups of its substrates (Zhou et al., 1998; Pruett et al., 2003; Azzi et al., 2004; Clark, Davulcu & Chapman, 2012). As expected for an integral enzyme, the nonsynonymous substitutions we observed do not occur at these specific sites, though they may still influence its activity. APBP becomes phosphorylated during murine sperm capacitation and co-localizes with cholesterol during this process, but its specific function is unknown (Jha et al 2008). It does possess a Rossmann-like fold, indicating an enzymatic role. The nonsynonymous substitutions we observed in APBP occur in the Rossmann-like fold and are hypothesized to influence the activity of its binding site (Marshall et al., 2011).

Conclusions

A. socius complex crickets provide an excellent opportunity to identify patterns of evolution in speciation genes for two major reasons: speciation is incomplete as evidenced by ongoing gene flow in the field, and isolation is through a single type of reproductive isolation barrier (i.e., postmating prezygotic phenotypes). Therefore we looked for genes that contribute to postmating prezygotic isolation and exhibit fixed, or nearly fixed, nonsynonymous substitutions between species as putative speciation genes. We find that when speciation is ongoing, standard population genetics analyses based on θ and ω values are unable to detect signatures of positive selection contributing to fixed differences between species because insufficient time has passed for nucleotide variation to accumulate. Instead, hypothesis testing based on haplotype networks and gene trees proved to be more powerful at identifying putative postmating prezygotic isolation genes with fixed nonsynonymous substitutions between both pairs of species that may have led to speciation.

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Table 1(on next page)

Nucleotide variation within each *A. socius* complex species

Table 1. Nucleotide variation within each *A. socius* complex species with Tajima’s *D*-values. Fu and Li’s *D*-values were similar (not shown)

Table 1. Nucleotide variation within each *A. socius* complex species with Tajima's *D*-values. Fu and Li's *D*-values were similar (not shown)

		within *A. fasciatus*					within *A. socius*					within *A. sp. nov.* Tex				
Gene	Length	n	π_s	π_a	θ_{fas}	*D*	n	π_s	π_a	θ_{soc}	*D*	n	π_s	π_a	θ_{Tex}	*D*
AK	1173	9	0.002	<0.001	0.001	0.975	15	0.004	0.001	0.002	-1.316	6	0.003	0.001	0.002	0.355
APBP	705	9	0.001	0	0.001	-1.088	15	0.005	0	0.001	-0.334	8	0	0	0	n/a
EJAC-SP	726	9	0	0	0	n/a	16	0.001	<0.001	0.001	-1.311	9	0.003	0	0.001	1.401
GOT	1122	9	0.002	0	<0.001	0.986	17	0	0	0	n/a	9	0.005	0.001	0.002	0.578
SPAG6	426	9	0	0	0	n/a	17	0	0	0	n/a	8	0.005	0	0.001	1.167
SPI	315	9	0.007	0	0.002	-1.362	16	0	0	0	n/a	9	0.008	0.001	0.002	0.196
ACG69	414	9	0.005	0.004	0.007	-1.286	14	0.021	0.009	0.011	0.264	7	0	0	0	n/a

Table 2(on next page)

Nucelotide variation at each branching node of the *A. socius* complex species tree.

Table 2. Nucelotide variation at each branching node of the *A. socius* complex species tree. (P_N: nonsynonymous polymorphisms; P_S: synonymous polymorphisms; D_N: nonsynonymous fixations; D_S: synonymous fixations; κ_s: rate of nonsynonymous substitutions per nonsynonymous site; κ_a: rate of synonymous substitutions per synonymous site; $\omega = \kappa_a / \kappa_s$)

Table 2. Nucelotide variation at each branching node of the *A. socius* complex species tree. (P_N: nonsynonymous polymorphisms; P_S: synonymous polymorphisms; D_N: nonsynonymous fixations; D_S: synonymous fixations; κ_s: rate of nonsynonymous substitutions per nonsynonymous site; κ_a: rate of synonymous substitutions per synonymous site; $\omega = \kappa_\mathrm{a} / \kappa_\mathrm{s}$)

		between fas & (soc+Tex)							between soc & Tex						
Gene	Length	P_N	P_S	D_N	D_S	κ_s	κ_a	ω	P_N	P_S	D_N	D_S	κ_s	κ_a	ω
AK	1173	3	12	2	0	0.006	0.003	0.557	2	9	1	2	0.011	0.002	0.206
APBP	705	1	4	1	0	0.005	0.003	0.523	0	3	1	0	0.007	0.002	0.278
EJAC-SP	726	1	3	0	0	0.008	0.001	0.131	1	2	0	1	0.014	0.002	0.123
GOT	1122	3	7	1	1	0.011	0.002	0.142	3	4	0	2	0.011	0.001	0.116
SPAG6	426	0	1	0	2	0.029	0	0	0	1	0	0	0.004	0	0
SPI	315	3	4	0	0	0.02	0.003	0.15	1	1	2	3	0.054	0.009	0.158
ACG69	414	7	6	0	0	0.024	0.009	0.374	7	6	0	0	0.021	0.007	0.317

Table 3(on next page)

Tests of lineage-specific positive selection

Table 3. Tests of lineage-specific positive selection that detects different ω classes along branches of a gene tree. The model with best c-AIC score is shown.

Table 3. Tests of lineage-specific positive selection that detects different ω classes along branches of a gene tree. The model with best c-AIC score is shown.

	Best model found by GA Branch		
Gene	c-AIC	Classes	ω classes
AK	3539.54	3	1: 0, 2: 0.148, 3: >>1
APBP	2018.52	2	1: 0, 2: >>1
EJAC-SP	2068.19	1	1: 0.079
GOT	3218.71	2	1: 0, 2: 0.545
SPAG6	1208.88	1	1: <0.001
SPI	955.82	2	1: 0, 2: 0.822
ACG69	1377	2	1: 0.081, 2: >>1

Table 4(on next page)

Genealogical sorting index values based on individual gene trees

Table 4. Genealogical sorting index values based on individual gene trees (see Supplementary Figure 1). Values range from zero (complete polyphyly) to one (complete monophyly).

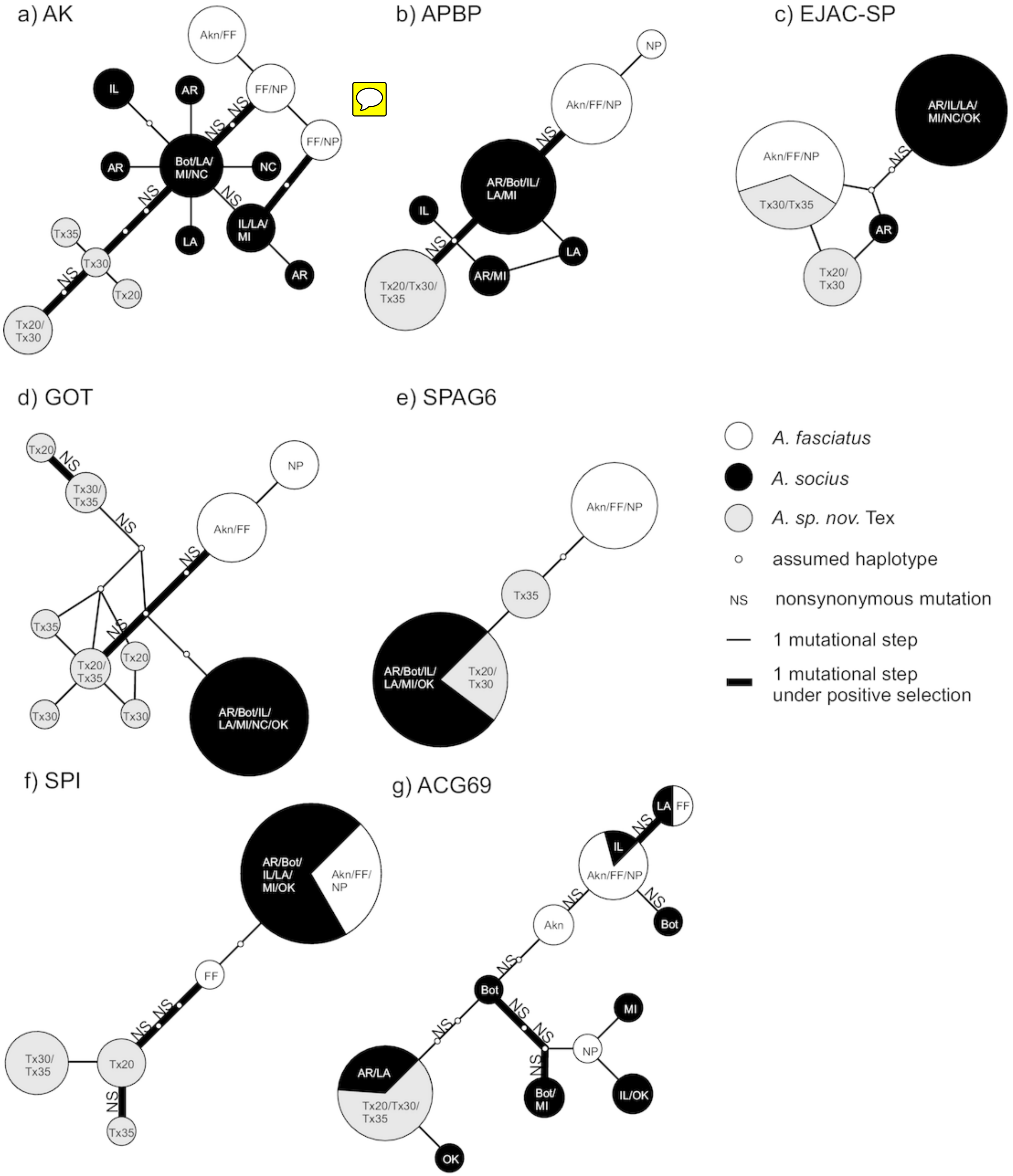
Table 4. Genealogical sorting index values based on individual gene trees (see Supplementary Figure 1). Values range from zero (complete polyphyly) to one (complete monophyly).

Gene	gsi-fas	P_{perm}	gsi-soc	P_{perm}	gsi-Tex	P_{perm}
AK	0.956	<0.001	0.919	<0.001	0.906	<0.001
APBP	1	<0.001	0.849	<0.001	1	<0.001
EJACSP	0.663	<0.001	0.732	<0.001	0.728	<0.001
GOT	0.919	<0.001	0.630	<0.001	0.934	<0.001
SPAG6	0.956	<0.001	0.670	<0.001	0.753	<0.001
SPI	0.140	0.001	0.339	<0.001	0.934	<0.001
ACG69	0.596	<0.001	0.05	0.644	0.917	<0.001

1

Statistical parsimony haplotype networks for all 7 genes from allopatric individual only

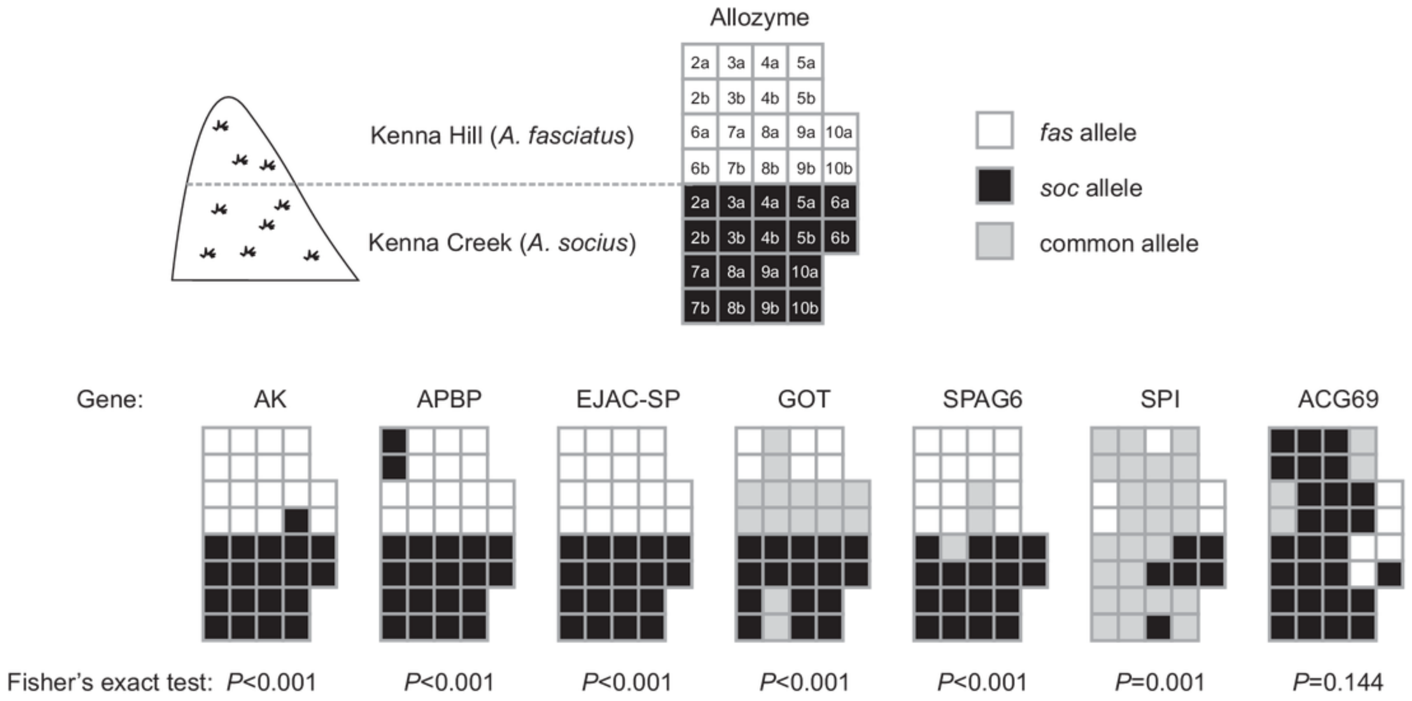
Figure 1. Statistical parsimony haplotype networks for all 7 genes (a-e: test; f-g: control) from allopatric individuals only, with nonsynonymous substitutions and mutational steps with elevated ω indicated. When more than two rate classes were detected (AK, see Table 1), only the largest rate class is indicated as a mutational step under positive selection. Population abbreviations are as in the main text.


a) AK
Akn/FF
FF/NP
FF/NP
IL
AR
AR
Bot/LA/MI/NC
NC
LA
IL/LA/MI
AR
Tx35
Tx30
Tx20
Tx20/Tx30
NS
b) APBP
NP
Akn/FF/NP
AR/Bot/IL/LA/MI
IL
LA
AR/MI
Tx20/Tx30/Tx35
NS
c) EJAC-SP
AR/IL/LA/MI/NC/OK
Akn/FF/NP
Tx30/Tx35
AR
Tx20/Tx30
NS
d) GOT
Tx20
Tx30/Tx35
NP
Akn/FF
Tx35
Tx20/Tx35
Tx20
Tx30
Tx30
AR/Bot/IL/LA/MI/NC/OK
NS
e) SPAG6
Akn/FF/NP
Tx35
AR/Bot/IL/LA/MI/OK
Tx20/Tx30
A. fasciatus
A. socius
A. sp. nov. Tex
assumed haplotype
NS nonsynonymous mutation
1 mutational step
1 mutational step under positive selection
f) SPI
AR/Bot/IL/LA/MI/OK
Akn/FF/NP
FF
Tx20
Tx30/Tx35
Tx35
NS
g) ACG69
LA
FF
IL
Akn/FF/NP
Akn
Bot
Bot
MI
NP
Bot/MI
IL/OK
AR/LA
Tx20/Tx30/Tx35
OK
NS

2

Distribution of species-specific vs. common (shared) alleles within the *A. fasciatus* and *A. socius* contact zone in Kenna, WV

Figure 2. Distribution of species-specific vs. common (shared) alleles within the *A. fasciatus* and *A. socius* contact zone in Kenna, WV. Nine individuals each with allozyme identities of pure (homozygous) *A. fasciatus* and *A. socius* had varying patterns of allelic identities for the seven genes. Numbers (2-9) indicate the sampled individual and letters (a & b) indicate the alleles within each individual.


Allozyme
2a 3a 4a 5a
2b 3b 4b 5b
6a 7a 8a 9a 10a
6b 7b 8b 9b 10b
2a 3a 4a 5a 6a
2b 3b 4b 5b 6b
7a 8a 9a 10a
7b 8b 9b 10b
Kenna Hill (*A. fasciatus*)
Kenna Creek (*A. socius*)
fas allele
soc allele
common allele
Gene:
AK
APBP
EJAC-SP
GOT
SPAG6
SPI
ACG69
Fisher's exact test: *P*<0.001
P<0.001
P<0.001
P<0.001
P<0.001
P=0.001
P=0.144