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

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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.

1 **Sorted gene genealogies and species-specific nonsynonymous substitutions point to putative**

2 **postmating prezygotic isolation genes**

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20 PopSets 372477483 (AK), 372477513 (APBP), 372477527 (EJAC-SP), 372477535 (GOT),

21 372477555 (SPAG6), 372477561 (SPI), 372477571 (ACG69).

23   **Abstract**

24   Not all genes contribute equally to reproductive isolation. In the *Allonemobius socius* complex of

25   crickets, reproductive isolation is primarily accomplished via postmating prezygotic barriers. We

26   show that two ejaculate protein-coding genes exhibit patterns of evolution consistent with a

27   putative role as speciation genes. Both genes express male ejaculate proteins transferred to

28   females during copulation and were previously identified through comparative proteomics. We

29   found gene genealogies indicating advanced degrees of lineage sorting, and fixed

30   nonsynonymous substitutions and elevated ω values on the mutational steps separating species,

31   between both pairs of species, on the haplotype networks of these genes compared to other

32   candidate and control genes. At a contact zone between two members of the species complex,

33   these genes maintained species-specificity of alleles despite ongoing gene flow. The putative

34   speciation genes *arginine kinase* (AK) and *apolipoprotein A-1 binding protein* (APBP) are two

35   of the first examples of sperm maturation, capacitation, and motility related proteins that show

36   evidence of fixed nonsynonymous substitutions between species-specific alleles that may lead to

37   reproductive isolation. Our results show that when speciation is ongoing and insufficient time

38   has passed for nucleotide variation to accumulate, hypothesis testing based on haplotype

39   networks and gene trees are more powerful than sequence-based population genetic metrics at

40   detecting signatures of positive selection that may have led to speciation.

Introduction

44 Not all genes contribute equally to reproductive isolation during speciation. 'Speciation' (Wu,

45 2001; Wu & Ting, 2004; Nosil & Schluter, 2011), 'isolation' (Rieseberg, Church & Morjan,

46 2004), or 'barrier' (Noor & Feder, 2006) genes are expected to show very different patterns of

47 evolution compared to genes that are not directly involved in reproductive isolation when species

48 are still undergoing lineage sorting (Wu 2001). Therefore we expect to find putative speciation

49 genes among those genes that become fixed for alternative alleles within each incipient species

50 early in the process of divergence, with said alleles rarely crossing the species boundary in

51 sympatry (Ting, Tsaur & Wu, 2000; Dopman et al., 2005).

52   Rapidly evolving reproductive proteins that can affect fertilization success have an

53 important role in the evolution of postmating prezygotic reproductive isolation. Many

54 reproductive genes are known to evolve rapidly in a variety of organisms (Civetta & Singh, 1998;

55 Swanson & Vacquier, 2002; Clark, Aagaard & Swanson, 2006; Panhuis & Swanson, 2006;

56 Snook et al., 2009). In *Drosophila* where some of the most extensive work has been done, genes

57 that show male-biased expression evolve faster compared to female-biased and somatically

58 expressed genes (Zhang, Hambuch & Parsch, 2004; Zhang & Parsch, 2005; Metta et al., 2006;

59 Pröschel, Zhang & Parsch, 2006; Haerty et al., 2007), and seminal fluid proteins in particular

60 tend to show an excess of nonsynonymous substitutions (Begun et al., 2000; Swanson et al.,

61 2001; Wagstaff & Begun, 2005; Almeida & DeSalle, 2008). Similar patterns have also been

62 observed in mice and primates (Clark & Swanson, 2005; Karn et al., 2008; Ramm et al., 2008;

63 Turner, Chuong & Hoekstra, 2008; Dean et al., 2009). Using a proteomics approach on insect

64 spermatophores to isolate male reproductive protein coding-genes that can directly interact with

65 female counterparts has proved to be an efficient way of narrowing prospects in the search for

66 putative speciation genes (Andrés, Maroja & Harrison, 2008; Marshall et al., 2011).

67   The male ejaculate proteome comprises sperm-expressed proteins and seminal fluid

68 proteins. Sperm not only contribute half of the diploid genome, but are also involved in sperm-

69 egg interactions including egg activation and deliver paternal factors during fertilization (Dorus

70 et al., 2006). Seminal fluid proteins, the majority of which are produced by male accessory

71 glands, contain conserved functional classes of peptides and pro-hormones that are involved in

72 sperm binding, proteolysis, lipid metabolism, and immune function (Mueller et al., 2004;

73 Chapman & Davies, 2004; Poiani, 2006; Avila et al., 2011). Once transferred into the female

74 reproductive tract, these proteins can initiate a wide-range of physiological functions including

75 increased egg production and oviposition, decreased receptivity, decreased lifespan, and

76 increased feeding in females (reviewed in (Avila et al., 2011). The interacting female

77 counterparts to these ejaculate proteins (EPs) are not well known (Ram, Ji & Wolfner, 2005;

78 Ram & Wolfner, 2007; Snook et al., 2009) though genomic data is proving to be invaluable for

79 identifying candidates (Findlay et al., 2014). The evolution of EPs has been hypothesized to be

80 driven by one or more processes including female sperm preference, sperm competition, and

81 sexual conflict (Mueller et al., 2004; Snook et al., 2009). Here, we show through multiple lines

82 of evidence that two EP-coding genes in the *Allonemobius socius* complex of crickets show

83 patterns of molecular evolution and gene genealogies consistent with a putative role as speciation

84 genes.

85   The *A. socius* complex of ground crickets, *A. socius*, *A. fasciatus*, and *A. sp. nov.* Tex,

86 represents a powerful system to explore the hypothesized link between EP divergence and

87 reproductive isolation. Members of this complex are primarily isolated from one another by two

88   postmating, prezygotic phenotypes – conspecific sperm precedence (Gregory & Howard, 1994;

89   Howard et al., 1998a,b; Marshall, 2004) and the superior ability of conspecific males to induce

90   females to lay eggs (Gregory & Howard, 1993; Howard et al., 1998b). Two other compelling

91   features of this organismal system are species boundaries that remain intact in sympatry despite

92   some gene flow (Howard, 1986; Howard & Waring, 1991; Traylor et al., 2008) and the very

93   recent nature of divergence between these species (i.e., within the last 30,000 years; (Marshall,

94   2004, 2007). Indeed, divergence is so recent that few species-specific alleles have been identified;

95   for example, only 2 of 17 allozyme markers (Howard, 1983), 2 of 5,400 AFLP markers (Howard

96   et al., 2002), ~21 of 1,660 thorax proteins and ~33 of 922 ejaculate proteins (Marshall et al.,

97   2011) and 1 of 16 randomly chosen reproductive genes spanning >7,500 bp of coding sequence

98   (Marshall et al., *unpublished data*), yield evidence of species specificity. Taken together, the

99   above data suggest that while there is sufficient genetic divergence to produce reproductive

100   isolation and maintain species boundaries in sympatry, the vast majority of genes show no

101   evidence of divergence and thus, no lineage sorting. In all, the *A. socius* complex represents a

102   system whereby speciation is ongoing with relatively few genes contributing to the postmating,

103   prezygotic reproductive isolation between species. Therefore, if we can identify those ejaculate

104   and female reproductive tract genes that exhibit signatures of positive selection, and maintain

105   species-specificity in sympatry, we will gain insight into the genes that contribute to reproductive

106   isolation and ultimately are involved in driving speciation.

107   In this study, we expanded analyses from a previous study comparing EPs between the

108   species *A. socius* and *A. fasciatus* (Marshall et al., 2011) by including more genes and an

109   additional species, *A. sp. nov.* Tex (Traylor et al., 2008). Specifically, longer fragments of the

110   five original proteins (ACG69, AK, APBP, EJAC-SP, SPI) plus two additional EPs (GOT,

111 SPAG6) were compared for patterns of nucleotide variation, evidence of lineage-specific

112 positive selection and different degrees of lineage sorting, and species-specificity of alleles in the

113 contact zone between *A. socius* and *A. fasciatus*. These combined analyses point toward an

114 important role for some but not all examined EPs during the evolution of reproductive isolation

115 within this complex of crickets.

116

117 **Methods**

118 *Background*

119   Striped ground crickets of the *A. socius* complex inhabit moist grasslands across North

120 America and do not show significant habitat isolation (Howard 1986). The three species *A.*

121 *socius*, *A. fasciatus*, and *A. sp. nov.* Tex form two contact zones, one between *A. fasciatus* (north)

122 and *A. socius* (south) from Illinois to New Jersey (Howard & Waring, 1991), and one between *A.*

123 *sp. nov.* Tex (west) and *A. socius* (east) near the Louisiana – Texas state line (Traylor et al.,

124 2008). *A. fasciatus* and *A. socius* seem to have diverged from a common ancestor approximately

125 30,000 years ago, and *A. sp. nov.* Tex seems to have subsequently diverged from *A. socius*

126 approximately 24,000 years ago (Marshall, 2004, 2007). They have previously been shown to be

127 isolated primarily via postmating prezygotic reproductive isolation (Howard et al., 2002;

128 Marshall, 2004; Marshall & DiRienzo, 2012).

129

130 *Population and gene sampling*

131   Crickets were collected from each population and genotyped in the lab via allozymes

132 (Isocytrate dehydrogenase and Hexokinase) to determine species identity (Howard, 1983, 1986).

133 Sampling localities spanned the range of each species. *A. socius* populations were sampled near

134 Texarkana, AR (AR), Bottom, NC (Bot), Mt. Vernon, IL (IL), Pleasantville, NJ (Mi), Ruston,

135 LA (LA), Gastonia, NC (NC), and Ardmore, OK (OK). *A. fasciatus* populations were sampled

136 near Akron, OH (Akn), Frankfort, IL (FF), and New Paltz, NY (NP). *A. sp. nov.* Tex populations

137 were sampled near Terrell, TX (Tx20), Royse City, TX (Tx30), and Gainesville, TX (Tx35).

138 Contact zone populations of *A. fasciatus* and *A. socius* were sampled from two habitats at a

139 single location in Kenna, WV. *A. fasciatus* was collected from a hillside habitat, which we call

140 Kenna Hill (KH), and *A. socius* was collected along the base of hill near a creek which we call

141 Kenna Creek (KC). We did not have samples from the contact zone between *A. socius* and *A. sp.*

142 *nov.* Tex. General maintenance protocols followed Marshall et al (2009).

143  We dissected male accessory glands and testes from three individuals per allopatric

144 population and 9 individuals per contact zone population. cDNA was synthesized from each

145 tissue using RNA isolated via an Ambion RNAqueous-4PCR (#AM1914) kit and standard

146 protocols for 1st strand cDNA synthesis. General PCR and sequencing procedures followed

147 Marshall et al (2011). Standard PCR chemistry was followed with annealing temperatures

148 between 50-60 °C depending on individual primer melting temperatures (primers used are shown

149 in Supplementary Table 1). We compared nucleotide sequences of five candidate EP genes with

150 two control EP genes. Among the five candidate genes, two were chosen based on species-

151 specific proteome profiles (Marshall et al., 2011): 1) *arginine kinase* (AK), a phosphotransferase

152 enzyme expressed in the sperm that may be involved in sperm motility, capacitation or the

153 acrosome reaction (Strong & Ellington, 1993; Niksirat et al., 2015); 2) *apolipoprotein A-1*

154 *binding protein* (APBP), a phosphoprotein expressed in sperm and hypothesized to be involved

155 in sperm capacitation (Jha et al., 2008). Two were chosen based on previous sequencing data

156 showing species-specific molecular variation: 3) *ejaculate serine protease* (EJAC-SP), an

157   abundant accessory gland-expressed serine protease previously shown to be involved in the

158   induction of egg laying in successfully mated females (Marshall et al., 2009); 4) *aspartate*

159   *aminotransferase* (GOT), a pyridoxal-phospate-dependent aminotransferase expressed in the

160   testis and an allozyme historically used to diagnose species identity among *A. socius* complex

161   crickets (Howard, 1983, 1986). The last candidate gene was chosen based on a review of sperm

162   biology literature: 5) *sperm-associated antigen 6* (SPAG6), important for sperm flagellar motility

163   and the structural integrity of the central apparatus (Neilson et al., 1999; Sapiro et al., 2002).

164   The control genes had non species-specific proteome profiles (Marshall et al., 2011) and

165   were: 6) *serpine inhibitor* (SPI), a testis-expressed serine-type endopeptidase inhibitor; 7) *acg69*

166   (ACG69), a protein of unknown function expressed in the accessory glands. Sequences formatted

167   as haplotypes are available from NCBI GenBank PopSets 372477483 (AK), 372477513 (APBP),

168   372477527 (EJAC-SP), 372477535 (GOT), 372477555 (SPAG6), 372477561 (SPI), 372477571

169   (ACG69).

170

171   *Sequence evolution-based analyses*

172   Male biased genes have been shown to exhibit patterns of molecular evolution associated

173   with relaxed selective constraints or strong positive selection, such as higher rates of

174   nonsynonymous substitutions (Zhang, Hambuch & Parsch, 2004). We investigated multiple

175   metrics of molecular sequence evolution to test for evidence of selection and a departure from

176   neutral sequence evolution. We applied Tajima's D and Fu and Li's D tests to each gene to look

177   for evidence of departure from neutral allelic distributions within species (Tajima, 1989; Fu & Li,

178   1993). We compared polymorphism within species to divergence between species using HKA

179 tests (Hudson, Kreitman & Aguadé, 1987), and tested for differences in these ratios at each

180 branching node of the species tree.

181   Next, we compared polymorphism and divergence between synonymous and

182 nonsynonymous sites within each gene at each branching node of the species tree. We compared

183 ω, the rate ratios of synonymous substitutions per synonymous site κ_a (d_N) and nonsynonymous

184 substitutions per nonsynonymous site κ_s (d_S). We used McDonald-Kreitman tests to compare the

185 ratio of nonsynonymous to synonymous intraspecific polymorphisms to the ratio of

186 nonsynymous to synonymous fixed differences between species (McDonald, Kreitman & others,

187 1991). All tests were based on sequences aligned in BioEdit v.7.0.5.3 (Hall, 1999) and metrics

188 calculated using DnaSP v.5.10.01 (Librado & Rozas, 2009). For HKA tests, we used the program

189 hka provided by Jody Hey (Wang & Hey, 1996).

190

191 *Gene genealogy-based analyses*

192   Evolutionary relationships between species are tested with phylogenetic trees while

193 hypotheses of intraspecific relationships benefit from haplotype network-based approaches

194 (Posada & Crandall, 2001). Because our species are recently diverged, we used both tree-based

195 and haplotype network-based analyses to detect interesting patterns of gene evolution.

196   We used statistical parsimony haplotype networks (Templeton, Crandall & Sing, 1992) of

197 alleles from all three species to test for species-specificity of alleles. We used TCS (Clement,

198 Posada & Crandall, 2000) to generate the haplotype networks using only allopatric individuals.

199 Species-specific alleles were defined as those found only within each respective species.

200 Common or shared alleles were those observed in more than one species. Once alleles were

201 designated common or specific to a species, we turned to the *fasciatus - socius* contact zone. We

202   looked at nine individuals each of contact zone *A. fasciatus* and *A. socius* and determined what

203   types of allele these contact zone individuals possessed. As noted above, these individuals had

204   previously been designated as fully (homozygous) *A. fasciatus* or *A. socius* based on allozymes.

205   We used Fisher's exact tests with Freeman-Halton extensions for 2x3 contingency tables to

206   determine the probability of observing the distribution of *fas* vs. *soc* vs. shared alleles for each

207   gene.

208   We tested for lineage-specific positive selection on individual gene tree topologies using

209   the Genetic Algorithm (GA) Branch method (Pond & Frost, 2005) via the Datamonkey

210   webserver of the HyPhy package (Delport et al., 2010). GA Branch uses a genetic algorithm that

211   allows estimates of the nonsynonymous to synonymous substitution rate ratio ($d_N/d_S = \kappa_a/\kappa_s = \omega$)

212   to vary freely across branches within a phylogeny and compares models with different ω classes.

213   Only allopatric individuals were included in the analysis and neighbor-joining trees used by GA

214   Branch were generated natively within Datamonkey.

215   The genealogical sorting index (gsi) reflects the degree of lineage sorting of individual

216   gene genealogies that occurs during speciation, with values ranging from zero (complete

217   polyphyly) to 1 (complete monophyly) (Cummings, Neel & Shaw, 2008). We calculated gsi for

218   each gene using the online server (www.genealogicalsorting.org) with gene trees including both

219   allopatric and contact zone individuals. Sequences were phased in DnaSP prior to tree building

220   for all genes except APBP, which had no heterozygous individuals. Sorting is more difficult to

221   observe in phased data. We generated maximum likelihood gene trees with PhyML 3.0 (Guindon

222   et al., 2010) via the Mobyle server (Neron et al., 2009). We used nearest neighbor interchange

223   (NNI) tree search and HKY85 as our nucleotide substitution model. MEGA6 (Tamura et al.,

224   2013) was used to visualize these trees.

225

226 **Results**

227 *Sequence evolution-based analyses*

228   We found a general lack of both synonymous and nonsynonymous nucleotide variation

229 among all EP genes we investigated (Table 1). The Watterson estimator $\theta=4N_e\mu$ ranged from

230 0.001 to 0.011. Levels of θ in the EP candidate genes were approximately an order of magnitude

231 lower than the control genes, although this difference was not statistically significant (*fas* -

232 Mann-Whitney $U = 0$, $P = 0.051$; *soc* - Mann-Whitney $U = 4$, $P = 0.688$; Tex - Mann-Whitney U

233 $= 4.5$, $P = 0.845$). In no cases were Tajima's D or Fu and Li's D tests significantly different from

234 neutral expectations (Table 1) (all $P > 0.1$).

235   To compare polymorphism within species to divergence between species, we used a

236 standard multilocus HKA test and HKA outlier tests for each branching event. We included all

237 loci and performed 9999 rounds of coalescent simulations. The multilocus HKA test did not find

238 a significant departure from neutral expectations for the first branching event between *A.*

239 *fasciatus* and the two other species ($X^2 P = 0.916$). The outlier cell, which was *A. fasciatus* for

240 polymorphism in ACG69, was not significantly different in its pattern of polymorphism to

241 divergence ($P = 0.68$). The multilocus HKA test did find a significant departure from neutral

242 expectations for the second branching event between *A. socius* and *A. sp. nov.* Tex. ($X^2 P =$

243 0.012). However, the outlier cell, which was polymorphism at GOT in *A. sp. nov.* Tex., was not

244 significantly different in its pattern of polymorphism to divergence ($P = 0.06$).

245   We compared the rate ratios of nonsynonymous to synonymous substitutions $\omega = \kappa_a/\kappa_s$ at

246 each branching event of the species tree. When ω is larger than 1 and the nonsynonymous

247 substitution rate exceeds the synonymous substitution rate, positive or diversifying selection is

248   inferred. When ω is smaller than 1, negative or purifying selection is inferred. However, $\omega = 1$ is

249   recognized as a conservative threshold because the average κ_a is expected to be much smaller

250   than κ_s given the expectation of widespread purifying selection acting on functional genes

251   (Nielsen, 2001). Therefore the value $\omega = 0.5$ has been suggested as an alternate cutoff for the

252   detection of positive selection as subsequent analyses generally indicate that such genes are

253   indeed under positive selection (Swanson et al., 2004). In none of our genes did ω exceed 1, but

254   in the older split between *A. fasciatus* and the other two species, ω exceeded 0.5 for the genes

255   AK and APBP (Table 2).

256   We used McDonald-Kreitman tests to compare the ratio of nonsynonymous to

257   synonymous intraspecific polymorphisms (P_N/P_S) to the ratio of nonsynymous to synonymous

258   fixed differences between species (D_N/D_S). Not all genes had fixed nonsynonymous substitutions

259   between species and in these cases we were unable to apply the McDonald-Kreitman test. For

260   those genes that were testable, we did not find significant differences in D_N/D_S compared to

261   P_N/P_S at either branching event (Fisher's exact test $P = 0.07 \sim 1$) (Table 2).

262

263   *Gene genealogy-based analyses*

264   The statistical parsimony haplotype networks generated using allopatric individuals of all

265   three species showed the presence of only species-specific alleles in AK, APBP, and GOT, while

266   the other genes had alleles shared between two species each (Figure 1). Within the contact zone

267   between *A. fasciatus* and *A. socius*, AK, APBP, EJAC-SP and SPAG6 had upwards of 16

268   species-specific alleles out of 18 possible alleles (approximately 88%) (Figure 2). In comparison,

269   many GOT and SPI alleles were shared between the contact zone populations. Fisher's exact

270   tests indicated the allelic distributions were nonrandom for all genes except ACG69.

271   The GA Branch method detected elevated ω classes in all genes except EJAC-SP and

272   SPAG6 (Table 3). We mapped the substitution rate changes detected by GA Branch onto the

273   haplotype networks (Figure 1). In AK and APBP, elevated ω were detected on mutational steps

274   between both species pairs, between *A. fasciatus* and *A. socius* and between *A. socius* and *A. sp.*

275   *nov.* Tex, and were associated with one or more fixed nonsynonymous substitutions. In GOT and

276   SPI, elevated ω were detected on mutational steps between *A. fasciatus* and *A. sp. nov.* Tex, and

277   also within *A. sp. nov.* Tex. Elevated ω were detected on several branches in ACG69 (Figure 1).

278   Comparisons of genealogical sorting index values based on maximum likelihood gene

279   trees including all sampled individuals, both allopatric and contact zone, indicated that only AK

280   and APBP showed advanced lineage sorting for all three species (Table 4, Supplementary

281   Figures 1 & 2). Excluding *A. sp. nov.* Tex, which had high gsi-values overall most likely due to

282   its limited range and lack of data from its contact zone with *A. socius*, the two control genes were

283   unsorted across the species ranges. The remaining three candidate genes showed asymmetrical

284   lineage sorting.

285

286   **Discussion**

287   Many reproductive genes, and in particular those that are male biased, are known to

288   evolve rapidly, often exhibiting higher rates of nonsynonymous substitutions (Zhang, Hambuch

289   & Parsch, 2004), reduced codon usage bias (Hambuch & Parsch, 2005), and evidence suggesting

290   they are more likely to evolve by duplication (Ellegren & Parsch, 2007). However recent

291   divergence can hinder the application of many metrics of molecular evolution that rely on

292   sequence variation since not enough evolutionary time has passed to allow for differences to

293   accumulate between incipient species. Thus data from relatively recently (~30,000 years)

294   diverged species such as ours show a general lack of both synonymous and nonsynonymous

295   nucleotide variation among all investigated genes (Table 1 & 2). In addition, our estimates of

296   sequence variation were also at least an order of magnitude smaller compared to other known

297   estimates from accessory gland protein coding genes in various other species groups (Mueller et

298   al., 2005; Wagstaff & Begun, 2005; Almeida & DeSalle, 2008), including some *Gryllus* crickets

299   whose species are of roughly similar age (Andrés et al., 2006). Therefore, while relatively young

300   species offer an opportunity to observe the ongoing process of the genetics of species divergence,

301   attempting to identify putative speciation genes based on DNA sequences requires an approach

302   that takes into account gene trees and haplotype networks, along with species trees.

303   The ratio of nonsynonymous to synonymous substitution rates ω is commonly used to

304   detect signatures of selection acting upon protein coding genes (Yang & Bielawski, 2000;

305   Nielsen, 2001, 2005; Jensen, Wong & Aquadro, 2007). The original intended application of ω

306   was for the analysis of sequence evolution among distantly related species, though in practice, it

307   is not uncommonly applied to sequences between closely related populations of a species

308   (Kryazhimskiy & Plotkin, 2008). This turns out to be problematic because when sequence

309   evolution under selection was simulated over short evolutionary timescales, representative of

310   genetic variation segregating within a species, vs. long evolutionary timescales, intraspecific ω

311   behaved very differently from interspecific ω (Kryazhimskiy & Plotkin, 2008). In fact,

312   Kryazhimskiy and Plotkin show that under positive selection, $\omega = 1$ when selection was

313   moderate but showed asymptotic behavior and eventually decreased below 1 as the selection

314   coefficient increased. Over short timescales, its variance also increased as θ ($= 2N\mu$ in the paper)

315   became smaller, making it more difficult to accurately detect positive selection.

316   Another complication with using ω for short evolutionary timescales is that during initial

317   sequence divergence ω could be higher than expected because slightly deleterious

318   nonsynonymous mutations can persist in a population for generations due to genetic hitchhiking,

319   and a time lag before they are removed by purifying selection (Rocha et al., 2006). Based on

320   simulations, Rocha and colleagues showed this is why unexpectedly high ω values are frequently

321   observed among closely related (1 – 2% sequence divergence) bacteria species. As evolutionary

322   time progresses further, they show that synonymous mutations continued to accumulate and

323   exceeded the initial overrepresentation of nonsynonymous mutations. Therefore if ω is estimated

324   too soon after sequence divergence, one would expect to find high rates of false positive

325   detection.

326   Finally, even at longer evolutionary timescales the assumption that κ_s is effectively

327   neutral may need reconsideration, as a survey of 16 vertebrate genomes indicates that genes with

328   high ω are more strongly influenced by small κ_s rather than large κ_a (Wolf et al., 2009). Similar

329   patterns are observed in *Drosophila* species, where fast evolving genes show a negative

330   correlation between κ_a and synonymous site polymorphism π_s (Andolfatto, 2007; Macpherson et

331   al., 2007; Jensen & Bachtrog, 2010).Thus, the interaction between linkage and selection makes it

332   challenging to distinguish between recurrent positive selection, background selection, and Hill-

333   Robertson effects (Hill & Robertson, 1966; Charlesworth, 1994; Andolfatto, 2007; Charlesworth

334   et al., 2009). Therefore in order to detect adaptive evolution due to positive selection, applying

335   combinations of metrics including ω, Tajima's or Fu and Li's *D* and site frequency spectra, as

336   well as estimates of population size and recombination rates seems necessary (Nielsen, 2005).

337   We failed to detect positive selection based on ω, and estimates of *D* for all genes

338   compared here were not significantly different from neutral expectations (Tables 1 and 2). While

339   population bottlenecks are thus likely to have contributed to sequence variation patterns since

340   speciation in the *A. socius* complex is thought to coincide with glaciation history (Marshall, 2004,

341   2007), for our data sequence evolution-based tests are generally inconclusive as to demographic

342   reasons for why our genes might lack nucleotide variation.

343   Instead, tests based on individual gene genealogies and haplotype networks indicated AK

344   and APBP as putative speciation genes. Within the contact zone of *A. fasciatus* and *A. socius*,

345   AK, APBP, and EJAC-SP show significantly nonrandom patterns of allelic distributions and had

346   no shared alleles (Figure 2). When all allopatric and contact zone individuals were examined,

347   only the genealogies of AK and APBP indicated that these genes were more advanced in their

348   degree of lineage sorting compared to other candidate and control genes (Table 4, Supplementary

349   Figures 1 & 2). These patterns fit models of ongoing speciation in the face of gene flow, where

350   speciation genes are more likely to be fixed early on during lineage divergence (Wu, 2001).

351   Incomplete lineage sorting and introgression have been suggested to be confounding factors in

352   understanding speciation with ongoing gene flow (Machado & Hey, 2003; Broughton &

353   Harrison, 2003; Payseur, 2010). However, speciation genes are more likely to become fixed for

354   species-specific alleles early in the process of speciation and therefore are expected to be

355   relatively exempt from incomplete sorting and subject to reduced introgression. Similar patterns

356   have been observed in *Drosophila*, field crickets, and moths (Ting, Tsaur & Wu, 2000; Dopman

357   et al., 2005; Maroja, Andrés & Harrison, 2009; Andrés et al., 2013; Larson et al., 2013). It is

358   possible that these genes are not the direct targets but rather linked to targets of divergent

359   selection. Because both genes were identified through comparative proteomics (Marshall et al

360   2011) this seems relatively unlikely, but the genomic regions around these genes should be

361   investigated for evidence of selective sweeps to rule out this possibility.

362   Many studies of reproductive proteins report evidence of positive selection acting on a

363   subset of the genes examined, in both males (Begun et al., 2000; Swanson et al., 2001; Clark &

364   Swanson, 2005; Wagstaff & Begun, 2005; Andrés et al., 2006; Karn et al., 2008; Ramm et al.,

365   2008; Almeida & DeSalle, 2008; Walters & Harrison, 2010) and females (Swanson et al., 2004;

366   Panhuis & Swanson, 2006; Lawniczak & Begun, 2007; Prokupek et al., 2008; Kelleher &

367   Markow, 2009; Kelleher, Clark & Markow, 2011). However, there are few examples of adaptive

368   reproductive protein evolution leading to reproductive isolation outside of gamete recognition

369   proteins (e.g. (Geyer & Palumbi, 2003; McCartney & Lessios, 2004; Springer & Crespi, 2007).

370   Our putative speciation genes AK and APBP two of the first examples of sperm maturation and

371   capacitation related proteins that show evidence of fixed nonsynonymous substitutions between

372   species-specific alleles leading to reproductive isolation. In contrast to the other genes examined,

373   fixed nonsynonymous substitutions and elevated ω values only on the mutational steps

374   separating species on the haplotype network of APBP, and to a less exclusive extent in AK and

375   GOT, indicate that these EPs may have evolved under positive selection and contribute to the

376   reproductive isolation between these species (Table 3, Figure 1). We had previously observed

377   this pattern between *A. fasciatus* and *A. socius* for both AK and APBP (Marshall et al 2011), but

378   finding the same pattern in the mutational steps between *A. socius* and *A. sp. nov.* Tex with

379   different species-specific nonsynonymous substitutions emphasizes the potential importance of

380   these candidates.

381   Whether there are functional consequences to the species-specific nonsynonymous

382   substitutions in AK and APBP needs to be investigated further. Since both candidates were

383   identified by proteomic screens, we hypothesize that an interaction between each male EP and

384   the female reproductive tract during capacitation is responsible for the postmating prezygotic

385   isolation observed between the *A. socius* complex species. AK is a phosphagen kinase that

386   catalyzes ATP-regeneration and energy transport in invertebrates and some protozoa (Ellington,

387   2001; Noguchi, Sawada & Akazawa, 2001; Uda et al., 2006). Insects and other ecdysozoans

388   possess AK as their sole phosphagen system for cellular energy metabolism, and accordingly,

389   arginine phosphate and its phosphagen kinase AK are found primarily in muscles, but also in

390   sperm and compound eyes (Strong & Ellington, 1993; Kucharski & Maleszka, 1998; Ellington,

391   2001). The possible roles of AK as an EP can be related to sperm motility (Strong and Ellington

392   1993), capacitation, or the acrosome reaction (Niksirat et al., 2015). Two structural loops and

393   several active sites near them are the proposed interaction interface of AK with the guanidinium

394   groups of its substrates (Zhou et al., 1998; Pruett et al., 2003; Azzi et al., 2004; Clark, Davulcu &

395   Chapman, 2012). As expected for an integral enzyme, the nonsynonymous substitutions we

396   observed do not occur at these specific sites, though they may still influence its activity. APBP

397   becomes phosphorylated during murine sperm capacitation and co-localizes with cholesterol

398   during this process, but its specific function is unknown (Jha et al 2008). It does possess a

399   Rossmann-like fold, indicating an enzymatic role. The nonsynonymous substitutions we

400   observed in APBP occur in the Rossmann-like fold and are hypothesized to influence the activity

401   of its binding site (Marshall et al., 2011).

402

403   **Conclusions**

404   *A. socius* complex crickets provide an excellent opportunity to identify patterns of

405   evolution in speciation genes for two major reasons: speciation is incomplete as evidenced by

406   ongoing gene flow in the field, and isolation is through a single type of reproductive isolation

407   barrier (i.e., postmating prezygotic phenotypes). Therefore we looked for genes that contribute to

408   postmating prezygotic isolation and exhibit fixed, or nearly fixed, nonsynonymous substitutions

409   between species as putative speciation genes. We find that when speciation is ongoing, standard

410   population genetics analyses based on θ and ω values are unable to detect signatures of positive

411   selection contributing to fixed differences between species because insufficient time has passed

412   for nucleotide variation to accumulate. Instead, hypothesis testing based on haplotype networks

413   and gene trees proved to be more powerful at identifying putative postmating prezygotic

414   isolation genes with fixed nonsynonymous substitutions between both pairs of species that may

415   have led to speciation.

416

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700

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)

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

2 shown)

Gene	Length	within *A. fasciatus*					within *A. socius*					within *A. sp. nov.* Tex				
		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

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; $\omega = \kappa_a / \kappa_s$)

1 Table 2. Nucelotide variation at each branching node of the *A. socius* complex species tree. (P_N: nonsynonymous polymorphisms; P_S:

2 synonymous polymorphisms; D_N: nonsynonymous fixations; D_S: synonymous fixations; κ_s: rate of nonsynonymous substitutions per

3 nonsynonymous site; κ_a: rate of synonymous substitutions per synonymous site; $\omega = \kappa_a / \kappa_s$)

Gene	Length	between fas & (soc+Tex)							between soc & Tex						
		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

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.

1   Table 3. Tests of lineage-specific positive selection that detects different ω classes along

2   branches of a gene tree. The model with best c-AIC score is shown.

Gene	Best model found by GA Branch		
	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

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).

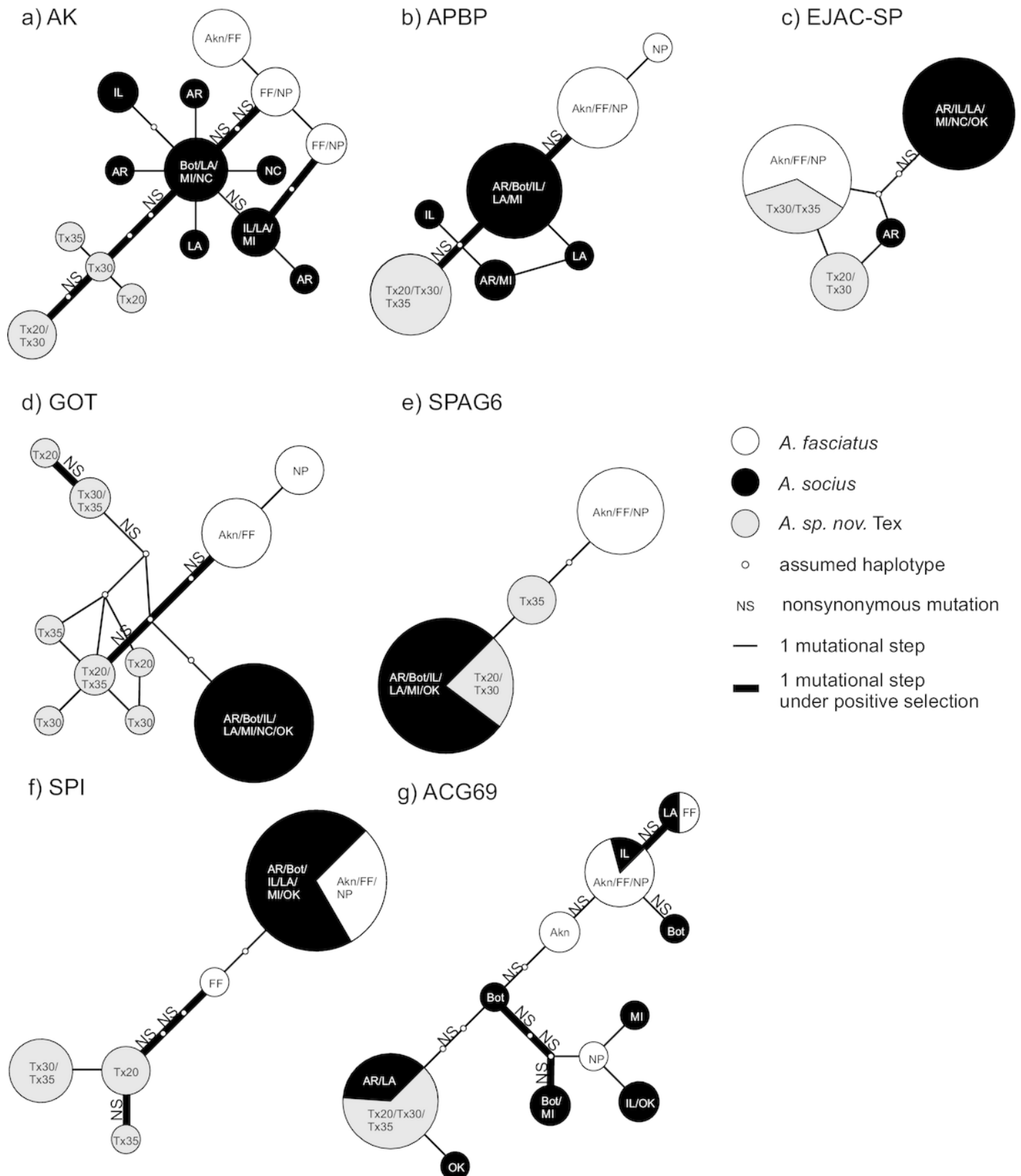
1   Table 4. Genealogical sorting index values based on individual gene trees (see Supplementary

2   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.

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.