Accelerated Evolution of Innate Immunity Proteins in Social Insects: Adaptive Evolution or Relaxed Constraint?

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Abstract

The genomes of eusocial insects have a reduced complement of immune genes—an unusual finding considering that sociality provides ideal conditions for disease transmission. The following three hypotheses have been invoked to explain this finding: 1) social insects are attacked by fewer pathogens, 2) social insects have effective behavioral or 3) novel molecular mechanisms for combating pathogens. At the molecular level, these hypotheses predict that canonical innate immune pathways experience a relaxation of selective constraint. A recent study of several innate immune genes in ants and bees showed a pattern of accelerated amino acid evolution, which is consistent with either positive selection or a relaxation of constraint. We studied the population genetics of innate immune genes in the honey bee Apis mellifera by partially sequencing 13 genes from the bee’s Toll pathway (~10.5 kb) and 20 randomly chosen genes (~16.5 kb) sequenced in 43 diploid workers. Relative to the random gene set, Toll pathway genes had significantly higher levels of amino acid replacement mutations segregating within A. mellifera and fixed between A. mellifera and A. cerana. However, levels of diversity and divergence at synonymous sites did not differ between the two gene sets. Although we detect strong signs of balancing selection on the pathogen recognition gene pgrp-sa, many of the genes in the Toll pathway show signatures of relaxed selective constraint. These results are consistent with the reduced complement of innate immune genes found in social insects and support the hypothesis that some aspect of eusociality renders canonical innate immunity superfluous.

Key words: innate immunity, positive selection, balancing selection, relaxed constraint, Apis mellifera.

Introduction

Canonical insect immune pathways are highly conserved and maintained as orthologs in most insects that have been fully sequenced (Rolf and Reynolds 2009). However, the genomes of eusocial insects, including several ants and the honey bee, Apis mellifera, have a reduced complement of immune genes relative to solitary insects (Evans et al. 2006; Smith CR, Smith CD, et al. 2011; Smith, Zimin, et al. 2011). For example, the genome of the honey bee contains approximately 66% fewer innate immune genes than found in Dipterans and 10–20% fewer immune genes than the solitary jewel wasp, Nasonia (Evans et al. 2006; Werren et al. 2010). The finding of a reduced complement of innate immune genes in social insects seems paradoxical considering that social insects live in dense societies that closely interact, providing ripe conditions for the spread of diseases and pathogens (Schmid-Hempel 2005; Naug and Smith 2007). For example, honey bee populations suffer from at least 29 known pathogens and pests (Evans and Schwarz 2011), which are partially responsible for large annual colony losses in both managed (Currie et al. 2010) and feral populations (Moritz et al. 2007).

Why do eusocial insects have a reduced complement of immune genes? Evans et al. (2006) proposed three hypotheses to explain this observation. First, eusocial insects have multiple effective forms of social/behavioral immunity (reviewed by Cremer et al. 2007; Evans and Spivak 2010). Second, social insects may have a reduced array of pathogens. Finally, innate immunity in social insects may rely on novel (i.e., noncanonical) molecular pathways. All of these hypotheses suggest that aspects of social insect biology render many canonical innate immune genes superfluous, which is predicted to cause a relaxation of purifying selection (i.e., reduced constraint) on such genes (Lynch and Conery 2000). For example, fewer pathogens may reduce the need for a high contingent of canonical immune genes and these nonessential genes will experience a relaxation of purifying selection resulting in an accumulation of nonsynonymous mutations relative to other genes. Left unchecked, this excess of nonsynonymous polymorphism will lead to an excess of nonsynonymous fixed differences between species relative to other genes in the genome.

Is there evidence of reduced constraint on intact innate immune genes in social insects? Viljakainen et al. (2009) recently documented accelerated amino acid evolution (i.e., high $d_N/d_S$) of several innate immune genes in both honey bees and ants, at much higher rates relative to Drosophila. The authors proposed two widely contrasting hypotheses to explain their results: 1) high pathogen pressure in social insects drive adaptive evolution of innate immune genes or 2) some aspects of social insect biology result in relaxed purifying selection acting on innate immune genes, which then accumulate amino acid changes at high rates. These two hypotheses predict high rates of protein evolution, but they are
readily distinguishable in light of polymorphism data (Nielsen 2005). Positive selection and reduced constraint are typified by accelerated amino acid replacements—nonsynonymous mutations are fixed at a higher rate. However, positive selection is expected to increase the rate of amino acid evolution (i.e., ratio of nonsynonymous to synonymous nucleotide divergence between species; Ka/Ks), while reducing the frequency of nonsynonymous polymorphisms (πNS) and linked synonymous (πS) polymorphisms (Nielsen 2005). Positive selection is also expected to skew the allele frequency spectrum relative to neutral expectations (Nei 1987; Tajima 1989). Positive selection can also increase levels of genetic differentiation (Fst) between populations if selection is geographically restricted (Beaumont 2005). In the case of reduced constraint, deleterious nonsynonymous mutations that are usually removed by purifying selection appear to evolve neutrally by contributing to both polymorphism within a species and divergence between species (Kimura 1983). Therefore, reduced constraint is expected to result in higher polymorphism rates (πNS) and nonsynonymous divergence (Ka), and allele-frequency spectra shifted toward higher frequency nonsynonymous mutations (Nei 1987; Tajima 1989). Balancing selection can also cause an elevated πNS and cause shifts in the allele-frequency spectra toward intermediate-frequency mutations. However, unlike reduced constraint, balancing selection is expected to increase πS and does not often increase divergence. Identifying patterns of diversity and divergence at synonymous and nonsynonymous sites is thereby crucial for understanding the evolutionary forces acting on canonical immune pathways in insects.

We undertook a large population genetic study of the honey bee’s Toll pathway to determine whether accelerated evolution of innate immune genes of social insects is consistent with positive selection or relaxed constraint; a test of the two hypotheses proposed by Viljakainen et al. (2009). The Toll pathway is a major component of the canonical immune system in solitary insects (Hoffmann 1995; Fritz et al. 2006) that recognizes and responds to bacteria and fungi (Tanji and Ip 2005; Evans et al. 2006; Valanne et al. 2011). We sequenced 10.5 kb of exonic sequence from 13 genes in the Toll-pathway in a large panel of A. mellifera workers, and in the closely related species A. cerana (for estimating divergence). We contrasted the population genetics of Toll-pathway genes to a data set comprised of 20 randomly chosen exons from the honey bee genome sequenced from the same individuals. We used the data set to examine whether the high rate of amino acid evolution previously found in innate immune genes are consistent with the outlined predictions of positive selection or relaxed purifying selection.

**Results**

**Differences between Toll Pathway Genes Relative to the Genome: Summary Statistics**

We validated previous observations of accelerated amino acid evolution of innate immune genes in the honey bee (Viljakainen et al. 2009): Toll pathway genes had significantly higher ratios of nonsynonymous to synonymous nucleotide divergence (Ka/Ks; table 1) between A. mellifera and A. cerana when compared with the random gene set (Global: F1,31 = 7.7, P = 0.009; Africa: P = 0.0016). This pattern is driven by Ka, which is more than three times higher in Toll genes than random genes (fig. 1A; Global: F1,31 = 11.6, P = 0.0018; Africa: P = 0.0060). The rate of divergence at synonymous sites (Ks) was not significantly different between Toll and random genes (fig. 1B; Global: F1,31 = 0.1, P = 0.90; Africa: P = 0.86). Analyses of nucleotide diversity mirrored those of nucleotide divergence. Relative to the random gene set, Toll genes had significantly higher nucleotide diversity at nonsynonymous sites (fig. 1C; Global: F1,31 = 9.2, P = 0.0048; Africa: P = 0.011) but not at synonymous sites (fig. 1D; Global: F1,31 = 0.17, P = 0.68; Africa: P = 0.65).

We examined whether higher estimates of Ka and πNS found in Toll genes were driven by a specific compartment of this pathway. We classified Toll genes based on predicted function (i.e., pathogen recognition receptor, signaling molecule, and antipathogen protein; table 1) and compared genetic diversity and divergence in these three groups with the random gene set. We found significant differences in πNS (table 1; Global: F3,29 = 4.3, P = 0.013; Africa: P = 0.028) across the four gene groups and a post-hoc test revealed that pathogen recognition genes have higher Ka and πNS than the random gene set (table 1; Tukey’s HSD [honestly significant difference]; Global: P = 0.01; Africa: P = 0.06). However, πS did not significantly differ among the four groups (table 1; Global: F3,29 = 0.41, P = 0.75; Africa: P = 0.77). We also found significant differences in Ka (table 1; Global: F3,29 = 6.4, P = 0.0018; Africa: P = 0.0049) but not Ks (table 1; Global: F3,29 = 0.45, P = 0.72; Africa: P = 0.76) between these functional groups, and a post-hoc analysis revealed that pathogen recognition genes have marginally significantly higher estimates of Ka than the random gene set (Tukey’s HSD; Global P = 0.053; Africa: P = 0.02). Although post-hoc tests showed that pathogen recognition genes have higher Ka and πNS, Toll genes still have significantly or marginally higher estimates of Ka and πNS relative to the random gene set when pathogen recognition genes were excluded (Ka – Global: F1,27 = 6.9, P = 0.014; Africa: P = 0.0061. πNS – Global: F1,27 = 4.8, P = 0.037; Africa: P = 0.059). This suggests that pathogen recognition genes are not the sole drivers of high Ka and πNS found in the Toll genes.

We did not find any significant differences between Toll genes and the random gene set for several parameters that summarize the shape of the allele frequency spectrum (table 1; Global Tajima’s D: F1,31 = 0.1034; F*, P = 0.09; and D*, P = 0.132; Africa Tajima’s D: P > 0.38 for all variables). Although a few Toll genes (table 1) had significantly negative estimates of D, F*, and D*, which are potential signs of purifying or positive selection, the proportion of genes with significant metrics of D, F*, or D* was not significantly different between Toll genes and the random gene set (Fisher’s exact test; P > 0.46 for all comparisons). However, if Toll genes are experiencing reduced constraint, we expect them to have higher estimates of Tajima’s D at nonsynonymous sites (D_{NSyn}) relative to the random set. We find that, on average,
Table 1. Summary Statistics for the Toll Pathway’s Antipathogen Proteins (AMP), Pathogen Recognition Receptors (PRR), and Signaling Molecules (SIG).

<table>
<thead>
<tr>
<th>Class</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>Sites</th>
<th>SNPs</th>
<th>π</th>
<th>πNS</th>
<th>Tajima’s D</th>
<th>D*</th>
<th>F*</th>
<th>MK</th>
<th>χ²</th>
<th>Ka</th>
<th>Ks</th>
<th>Ka/Ks</th>
<th>HKA P Value</th>
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<tr>
<td>AMP</td>
<td>GB10036</td>
<td>def2</td>
<td>275</td>
<td>6</td>
<td>2.31</td>
<td>9.1</td>
<td>0.22</td>
<td>-1.08</td>
<td>-2.61</td>
<td>-2.48</td>
<td>0.38</td>
<td>0.049</td>
<td>0.09</td>
<td>0.525</td>
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<td>GB10231</td>
<td>lys1</td>
<td>469</td>
<td>16</td>
<td>3.45</td>
<td>11.43</td>
<td>1.43</td>
<td>-1.4</td>
<td>-2.4</td>
<td>-2.42</td>
<td>0</td>
<td>0.001</td>
<td>0.009</td>
<td>0.123</td>
<td>&lt;0.01</td>
</tr>
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<td>AMP</td>
<td>GB18313</td>
<td>pro-PO</td>
<td>765</td>
<td>9</td>
<td>1.81</td>
<td>7.9</td>
<td>0</td>
<td>-0.6</td>
<td>-0.87</td>
<td>-0.92</td>
<td>1</td>
<td>0.017</td>
<td>0.103</td>
<td>0.158</td>
<td>0.26</td>
</tr>
<tr>
<td>AMP</td>
<td>GB18767</td>
<td>pro-POα</td>
<td>785</td>
<td>21</td>
<td>4.71</td>
<td>14.05</td>
<td>1.81</td>
<td>-0.68</td>
<td>-1.19</td>
<td>-1.19</td>
<td>0.69</td>
<td>0.02</td>
<td>0.099</td>
<td>0.191</td>
<td>0.25</td>
</tr>
<tr>
<td>PRR</td>
<td>GB19961</td>
<td>gnb1p1</td>
<td>360</td>
<td>9</td>
<td>1.47</td>
<td>3.76</td>
<td>0.74</td>
<td>-1.8</td>
<td>-0.87</td>
<td>-1.41</td>
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<td>0.011</td>
<td>0.061</td>
<td>0.168</td>
<td>0.56</td>
</tr>
<tr>
<td>PRR</td>
<td>GB17188</td>
<td>pgp-pc</td>
<td>1,311</td>
<td>14</td>
<td>2.31</td>
<td>7.44</td>
<td>0.84</td>
<td>-0.33</td>
<td>-1.2</td>
<td>-1.06</td>
<td>0.57</td>
<td>0.024</td>
<td>0.086</td>
<td>0.27</td>
<td>0.61</td>
</tr>
<tr>
<td>PRR</td>
<td>GB15371</td>
<td>pgp-sa</td>
<td>528</td>
<td>16</td>
<td>9.04</td>
<td>27.12</td>
<td>3.17</td>
<td>-0.049</td>
<td>-1.86</td>
<td>-1.54</td>
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<td>0.022</td>
<td>0.156</td>
<td>0.128</td>
<td>0.58</td>
</tr>
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<td>psh</td>
<td>954</td>
<td>17</td>
<td>3.02</td>
<td>8.2</td>
<td>1.47</td>
<td>-0.81</td>
<td>-2.14</td>
<td>-1.97</td>
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<td>dl</td>
<td>597</td>
<td>4</td>
<td>0.16</td>
<td>0.35</td>
<td>0.1</td>
<td>-1.8</td>
<td>-3.77</td>
<td>-3.69</td>
<td>1</td>
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<td>0.018</td>
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<td>myd88</td>
<td>1,275</td>
<td>15</td>
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<td>3.17</td>
<td>1.02</td>
<td>-1.36</td>
<td>-2.6</td>
<td>-2.57</td>
<td>0</td>
<td>0.015</td>
<td>0.088</td>
<td>0.159</td>
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<tr>
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<td>pli</td>
<td>809</td>
<td>8</td>
<td>0.53</td>
<td>2.13</td>
<td>0.04</td>
<td>-1.82</td>
<td>-3.46</td>
<td>-3.43</td>
<td>0</td>
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<td>0.116</td>
<td>0.114</td>
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</tr>
<tr>
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<td>spz</td>
<td>279</td>
<td>8</td>
<td>3.8</td>
<td>9.23</td>
<td>2.27</td>
<td>-0.83</td>
<td>0.48</td>
<td>0.045</td>
<td>0.69</td>
<td>0.044</td>
<td>0.078</td>
<td>0.545</td>
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</tr>
<tr>
<td>SIG</td>
<td>GB18520</td>
<td>tl</td>
<td>2,055</td>
<td>31</td>
<td>2.21</td>
<td>8.53</td>
<td>0.54</td>
<td>-0.84</td>
<td>-2.07</td>
<td>-1.92</td>
<td>0.45</td>
<td>0.014</td>
<td>0.122</td>
<td>0.105</td>
<td>0.88</td>
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<td>Toll set</td>
<td>Average</td>
<td>805</td>
<td>13</td>
<td>2.79</td>
<td>8.65</td>
<td>1.05</td>
<td>-1.04</td>
<td>-1.89</td>
<td>-1.89</td>
<td>0.69</td>
<td>0.02</td>
<td>0.093</td>
<td>0.21</td>
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<tr>
<td>95% range</td>
<td>1.8–3.8</td>
<td>5.6–11.6</td>
<td>0.63–1.4</td>
<td></td>
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<td></td>
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<tr>
<td>Random set</td>
<td>Average</td>
<td>812</td>
<td>17</td>
<td>2.53</td>
<td>10.22</td>
<td>0.31</td>
<td>-1.36</td>
<td>-2.27</td>
<td>-2.31</td>
<td>0.7</td>
<td>0.005</td>
<td>0.098</td>
<td>0.081</td>
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</tr>
<tr>
<td>95% range</td>
<td>1.3–3.8</td>
<td>4.7–15.7</td>
<td>0.13–0.49</td>
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</tbody>
</table>

Note.—Pairwise diversity estimates (π) reported as π × 10⁻³. Gene names from Evans et al. (2006). Underlined values are significant; summary statistics were estimated in the Global honey bee population, unless otherwise stated (see Materials and Methods).

**Fig. 1.** Toll-pathway genes have higher nucleotide divergence at nonsynonymous sites (A) but not at synonymous sites (B) relative to a random set of genes. Toll-pathway genes also have higher nucleotide diversity at nonsynonymous sites (C) but not at synonymous sites (D) relative to a random set of genes. *P < 0.05.
$D_{NS}$ is higher in the Toll genes relative to the random gene set (Global: one-tailed $P = 0.036$; Africa one-tailed $P = 0.07$), and this result becomes more significant once we remove genes with putative signs of selection (discussed later: def2, lys1, pgrp-sa, pli, dl; Global: one-tailed $P = 0.0079$; Africa one-tailed $P = 0.04$).

Estimates of global and pairwise genetic differentiation (Fst) did not significantly differ between Toll genes ($F_{st} = 0.24$) and the random ($F_{st} = 0.21$) gene set (Global Fst: $F_{1,26} = 0.51, P = 0.48$; Pairwise Fst: $P > 0.14$ for all comparisons; supplementary table S3, Supplementary Material online). Further, the proportion of mutations fixed by selection ($\alpha$) did not significantly differ between Toll genes and the random gene set ($F_{1,31} = 2.43, P = 0.13$), even when we excluded genes with $\alpha = 0$ from our analyses ($F_{1,12} = 0.008, P = 0.93$). None of the Toll genes examined herein had significant signs of selection based on the McDonald–Kreitman (MK) test. Finally, only one innate immune gene had a gene-wide signature of balancing selection after a 5% false discovery rate (FDR) correction (lys1; Hudson, Kreitman, and Aguade [HKA] test $\chi^2 = 17.73, P < 0.0001$).

**Gene-Centric Evolutionary Analyses on Toll-Pathway Genes**

By integrating results of tests of selection (e.g., MK and HKA) with estimates of the rate of amino acid evolution (Ka/Ks) relative to nonsynonymous diversity ($\pi_{NS}$) (fig. 2), we were able to highlight groups of genes with specific and consistent patterns of molecular evolution, as described later.

**Genes with Signs of Purifying Selection and Constraint**

Strong purifying selection is expected to result in low diversity and divergence at nonsynonymous sites, and consequently low Ka/Ks ratios (Nielsen et al. 2007). Both pli and dl have low nonsynonymous diversity and rates of amino acid evolution (fig. 2) and have very few polymorphic nonsynonymous mutations (an average of 1.25 per gene) that tend to be very rare in frequency ($<2\%$). Consequently, pli and dl have significantly negative estimates of Tajima's $D$, and Fu and Li's $F^*$ and $D^*$ (table 1). These lines of evidence are indicative of purifying selection acting on pli and dl.

**Genes with Signs of Positive Selection**

Positive selection is expected to increase Ka/Ks and reduce $\pi_{NS}$ relative to $\pi_S$ (Yang and Bielawski 2000; Nielsen 2005). Toll pathway genes did not exhibit significant signs of positive selection as indicated by the MK test (table 1). Nevertheless, the antipathogenic gene def2 showed patterns of molecular evolution that are consistent with positive selection: high Ka/Ks (0.525, the second highest among the 33 genes studied here) and low $\pi_{NS}$ relative to other immune genes (fig. 2). Because def2 is the smallest gene in our study (275 bp), we expect low statistical power for rejecting neutral evolution using the MK test (Begun et al. 2007; Andolfatto 2008). As such, we consider def2's accelerated amino acid evolution, and low $\pi_{NS}$ to represent putative signs of positive selection, which is consistent with previous studies of this gene in other social insects (Viljakainen and Pamilo 2005). The signaling gene spz also has high Ka/Ks, but its high $\pi_{NS}$ and $\pi_{NS}/\pi_S$ ratio are more consistent with relaxed constraint than adaptive evolution (fig. 2; discussed later).

**Genes with Signs of Balancing Selection**

Balancing selection acts to increase genetic diversity at functional nonsynonymous sites and nearby synonymous sites (Nielsen 2005; Charlesworth 2006), and also acts to reduce the ratio of interspecific to intraspecific variability (Nielsen 2005). By homogenizing allele frequencies, balancing selection also skews the allele frequency spectrum toward intermediate frequencies (Nielsen 2005; Weedall and Conway 2010), resulting in higher values of Tajima's $D$ (Tajima 1989). The single-exon gene lys1 showed promising signs of balancing selection, with both high $\pi_{NS}$ low Ka, and Ka/Ks, and a significant HKA test result when compared with the random gene set ($\chi^2 = 17.73, P < 0.0001$) and intronic set ($\chi^2 = 8.34, P = 0.0039$).

Although pgrp-sa's gene-wide HKA test was not significant, it also exhibited several signs of balancing selection, including an extremely high estimate of $\pi_{NS}$ (outside of the 95% confidence interval [CI] of both the random gene set and all Toll pathway genes; fig. 2) and an extremely high value of Tajima’s $D$ (pgrp-sa $D_{*} = -0.049$ vs. genomic average $D_{*} = -1.36$; table 1). Synonymous diversity of pgrp-sa was also extremely high relative to random and Toll genes (fig. 2). Because these data are highly suggestive of balancing selection, we investigated this gene in more depth by conducting analyses on functionally relevant protein domains (Sharma et al. 2008). We were able to thread the honey bee's PGRP-SA onto previously elucidated PGRP structures.

We observed that much of the elevated nonsynonymous diversity was localized at the PGRP-specific region of PGRP-SA (Global: $\pi_{NS} = 0.021$; Africa: $\pi_{NS} = 0.016$), which is two orders of magnitude more diverse than the rest of the gene (Global: $\pi_{NS} = 0.0030$; Africa: $\pi_{NS} = 0.00035$). We were able to detect significant signatures of balancing selection acting on the PGRP-specific domain using the HKA test; it has significantly more diversity and less divergence relative to the random gene set ($\chi^2 = 7.351, P = 0.0067$), other PGRP-SA protein domains ($P = 0.01$), and introns sequenced herein ($P = 0.04$).

**Genes with Signs of Relaxed Constraint**

Deleterious nonsynonymous mutations are removed via purifying selection, but if purifying selection is relaxed, nonsynonymous mutations can accumulate and increase in frequency, contributing to both polymorphism and divergence (Kimura 1983; Streifeld and Rausher 2007). We observed that many of Toll pathway genes sequenced herein have elevated $\pi_{NS}$ and Ka, but not $\pi_S$ and Ks, relative to the rest of the genome (figs. 1 and 2). Further, we observed a strong and significant positive relationship between $\pi_{NS}$ and Ka/Ks ratios ($r = 0.75, P = 0.013$) in immune genes after excluding those Toll pathway genes with putative signs of balancing and positive selection (pgrp-sa, lys1, and def2); genes with high estimates of $\pi_{NS}$ also exhibited high estimates of Ka, and consequently, Ka/Ks. We did not detect a relationship.
between \( \pi_{NS} \) and Ka/Ks ratios in the random gene set \( (r = 0.105, P = 0.66) \). No genes in the random set had significant signs of positive selection based on MK tests (FDR < 0.05) and a single gene had putative signs of positive selection based on high Ka/Ks and low \( \pi_{NS} \); removing this gene had no effect on the previously observed nonsignificant correlation between \( \pi_{NS} \) and Ka/Ks ratios in the random gene set \( (r = 0.25, P = 0.28) \). Finally, we note that Toll pathway genes with signs of relaxed constraint were slightly but not significantly larger than Toll pathway genes with signs of negative, positive, or balancing selection (i.e., \textit{def2}, \textit{lys1}, \textit{pggrp-sa}, \textit{pli}, \textit{dl} vs. other Toll genes; Student’s t test, \( P = 0.133 \)), which suggests that signatures of relaxed constraint on the Toll pathway were not primarily observed in a subset of small genes with low statistical power to detect selection.

**Discussion**

**Adaptive Evolution of Innate Immunity and the Need to Go Beyond \( dn/ds \)**

The use of both polymorphism data and divergence data are critical for understanding the evolutionary forces acting on gene sequences (Nielsen 2005; Kryazhimskiy and Plotkin 2008). Although some studies interpret high ratios of \( dn/ds \) as a putative sign for positive selection (e.g., supplementary table S4, Supplementary Material online), we note, as others have, that the \( dn/ds \) approach is most useful when used in tandem with polymorphism data because both positive selection and reduced constraint can elevate the rate of amino acid evolution (Yang and Bielawski 2000; Nielsen 2005). Population-level sampling also allows for the use of a host of statistical tests that can be used to identify loci under selection. This includes the robust MK class of tests (Hudson et al. 1987; McDonald and Kreitman 1991; Eilertson et al. 2012), as well as tests utilizing the shape of the allele-frequency spectrum (Nei 1987; Tajima 1989; Fu and Li 1993) and outlier patterns of differentiation between populations (Beaumont 2005). Together, these data can be used to generate total evidence-based inferences regarding the evolutionary forces acting on specific loci, as we summarize later.

Total evidence-based approaches have been used in studies on termite and ant antipathogen proteins (Viljakainen and Pamilo 2008; Bulmer et al. 2010) and many Dipteran immune genes (Lazzaro and Clark 2003; Schlenke and Begun 2003; Levine and Begun 2007; Cohuet et al. 2008). Studies on social insects include those of Bulmer et al. (2010) who used populations of two termite species and found positive selection acting on pathogen-recognition proteins and antipathogen proteins using data on polymorphisms, Ka/Ks, and...
the MK test. Similarly, Viljakainen and Pamilo (2008) sequenced defensins in 91 ants from 27 species and used both polymorphism data as well as dn/ds to find evidence of positive selection. Schlenke and Begun (2003), Cohuet et al. (2008), and Levine and Begun (2007) have collectively investigated over 80 Dipteran immune genes using MK tests, Ka/Ks, and patterns of population-level polymorphism to find positive selection acting on several genes including def2, Relish, and Tep1 (supplementary table S4, Supplementary Material online). In contrast to the earlier mentioned studies, our work suggests that in honey bees, positive selection plays a limited role in the evolution of innate immune genes. We found putative signs of positive selection on only a single gene, def2, which exhibits both high Ka/Ks and low πNS. Our findings along with others (Viljakainen and Pamilo 2005; Viljakainen and Pamilo 2008; Gao and Zhu 2010) suggest that def2 plays an important role in host-pathogen arms races in the Hymenoptera.

Surprisingly, we found signs of balancing selection acting on lys1 and pgrp-sa in the honey bee. Balancing selection has been found to act on innate immune genes in mammals (Hedrick 1998; Aguilar et al. 2004) and plants (Van der Hoorn et al. 2002), but is uncommon in insects (Rottscchafer et al. 2011). Signatures of balancing selection on pgrp-sa, a pathogen recognition receptor, are particularly intriguing because genes involved in pathogen-recognition in mammals and plants often evolve via balancing selection. In the case of mammals, highly diverse pathogen recognition genes allow for the recognition of a diverse array of pathogens (Hedrick 1998; Aguilar et al. 2004). Balancing selection on these genes is restricted to the pathogen recognition domains, the site of pathogen binding. This is not the case for PGRP-SA in honey bees or in other insects (Jiggins and Hurst 2003; Little and Cobbe 2005; Sackton et al. 2007; Lazzaro 2008). The unique signatures of balancing selection on honey bee PGRP-SA were localized at the PGRP-specific site, a protein domain that interacts with downstream signaling molecules or other recognition receptors and initiates the Toll signaling cascade (Guan et al. 2004, 2005; Zaidman-Remy et al. 2006). It is unclear how increased diversity in this site contributes to the function of PGRP-SA in honey bees; however, variation in the sequence of the PGRP-specific region across taxa is hypothesized to result in different ligand-binding efficiencies (Guan et al. 2005). Increased diversity at this domain may allow PGRP-SA to bind alternative downstream targets depending on the pathogen or stressor present. Alternatively, increased functional diversity at this domain may be a response to bacterial interference. Gram-negative bacteria, which are recognized by PGRPs (Guan et al. 2005), can release molecules that interfere with downstream signaling (Worrall et al. 2011) by potentially interfering with PGRP-SA’s binding affinity. Selection could possibly act to reduce binding affinity of PGRP-SA to interfering pathogenic proteins in a frequency-dependent manner. This scenario is similar to the evolution of R genes in plants (Van der Hoorn et al. 2002). More research is needed to understand the underlying causes of balancing selection on the honey bee’s PGRP-SA.

Relaxed Constraint Best Explains Accelerated Amino Acid Evolution of Innate Immune Genes in Honey Bees

The population genetics of Toll pathway genes stand in stark contrast relative to orthologs in Anopheles and Drosophila that often show reduced genetic variation relative to nonimmune genes because of a combination of purifying and positive selection (Schlenke and Begun 2003; Parmakelis et al. 2008). Viljakainen et al. (2009) previously suggested that relaxed purifying selection can be responsible for the substantially higher estimates of amino acid evolution of innate immune genes in social insects (relative to Drosophila), and our population genetic data support their proposition. The majority of Toll pathway genes studied herein (8 out of 13) show signatures of relaxed purifying selection including high rates of nonsynonymous polymorphisms, high rates of nonsynonymous divergence, typical rates of polymorphism and divergence at silent sites, and an increased allele frequency of nonsynonymous mutations relative to other genes. The alternative hypothesis that high rates of amino acid evolution in Toll pathway genes are caused by positive selection propelled by host–pathogen arms races is not well supported as most Toll pathway gene lack significant signatures of selection (i.e., MK and HKA tests), and because the high rates of nonsynonymous but not synonymous mutations in such genes are not consistent with positive selection. Although we observe strong signatures of balancing selection acting on pgrp-sa and lys1, the other Toll pathway genes do not exhibit signs of balancing selection, such as high πS and Tajima’s D, and a low ratio of interspecific to intraspecific variability (Nielsen 2005; Charlesworth 2006; Gos and Wright 2008; Weedall and Conway 2010). We note that both the random gene set and the Toll pathway gene set were sequenced in the same individuals from the same populations using identical methods, and that the number of sequenced sites per gene did not significantly differ between the two gene sets (analysis of variance [ANOVA], P = 0.954). As such, our finding of substantially higher rates of nonsynonymous diversity in Toll pathway genes—representing strong evidence for relaxed constraint—is unlikely to be caused by differences in methodology or sample size between the two data sets.

Implications of Relaxed Constraint on Innate Immune Genes in the Honey Bee

The genomes of several eusocial insects contain fewer innate immune genes relative to solitary insects (Evans et al. 2006; Smith CR, Smith CD, et al. 2011; Smith, Zimin, et al. 2011). Such reductions suggest that the shift to eusociality either reduced pathogen loads experienced by eusocial insects, and/or provided them with more effective or less costly means to combat pathogens than the canonical innate immune pathways (Evans et al. 2006). These hypotheses predict a relaxation of purifying selection acting on innate immune genes. Our study suggests that innate immune genes in the honey bee mostly evolve through relaxed selective constraint. Additionally, our results suggest that the evolutionary forces that
caused reductions in the number of innate immune genes in the honey bee are still acting (at least until recently) (Cui et al. 2011; Yang et al. 2011; Dainat et al. 2012). It is important to note that the Toll pathway genes studied herein are known to be expressed during immune challenges (Evans and Pettis 2005; Decanini et al. 2007; Chan et al. 2009). Although this may seem at odds with population genetic evidence for reduced selective constraint, pseudogenes for example, can still be expressed until mutation erodes regulatory sequences (Cui et al. 2011; Yang et al. 2011). Further, it is important to note that expression during immune cascades (Evans and Pettis 2005; Decanini et al. 2007; Chan et al. 2009; Huang et al. 2012) does not on its own provide evidence that the Toll pathway genes studied herein are functionally contributing to innate immunity. Only a few innate immune genes have been functionally characterized in social insects (Casteels et al. 1990; Bulmer and Crozier 2004; Aronstein and Saldivar 2005; Schluns and Crozier 2007); more work is needed to determine whether the Toll pathway genes with signs of relaxed constraint are essential to honey bee immunity.

The observed signs of relaxed selective constraint acting on innate immune genes in the honey bee may also be present in other social insects, considering both the reduction in innate immune genes and the accelerated evolutionary rate of such genes in ants (Viljakainen et al. 2009; Smith CR, Smith CD, et al. 2011). If the shift to eusociality renders some innate immune genes superfluous then we would predict marked differences in the number of innate immune genes, and in the evolutionary tempo of such genes, across social and solitary species of the Hymenoptera. The future availability of genomic data on phylogenetically independent eusocial and solitary species pairs will greatly enhance our understanding of the relationship between social behavior and the evolution of innate immunity.

Materials and Methods

Sampling, Gene Selection, and DNA Sequencing

In the old world, honey bees are divided into four major population groups found in Africa, Asia, East, and West/Northern Europe (Whitfield et al. 2006). These populations are characterized by a high degree of genetic differentiation between groups and very low levels of differentiation within groups (Whitfield et al. 2006). We sampled three major population groups of *A. mellifera* from Africa (N = 12 workers, 24 chromosomes per locus), East Europe (N = 10 workers, 20 chromosomes per locus), and West Europe (N = 12 workers, 24 chromosomes per locus); these populations represent relatively pure progenitor populations in the old world (Harpur et al. 2012). We also obtained bees from managed populations in Canada (N = 9 workers, 18 chromosomes per locus), which are admixed between East European and West European subspecies (Harpur et al. 2012); see Population Genetic Analyses for how samples from different populations were treated. Finally, we sampled one diploid worker of *A. cerana* from Thailand (2 chromosomes per locus) as an out-group for estimating sequence divergence. Nucleotide diversity of exons sequenced from *A. cerana* correlated with those of *A. mellifera* (rho = 0.646, P < 0.0001), but we did not formally analyze diversity in *A. cerana’s* immune genes because of the low statistical power (i.e., we only sampled two haplotypes; Pons and Chaouche 1995).

We targeted 13 genes in the honey bee’s Toll Pathway (supplementary table S1, Supplementary Material online) known to be expressed during immune challenges, suggesting a putative role in innate immunity (Evans et al. 2006). We sequenced a total of approximately 10.5 kb of exonic sequences from the chosen genes for each bee using polymerase chain reaction (PCR) primers (supplementary table S1, Supplementary Material online) designed from the honey bee’s official gene set (OGS1; Honeybee Genome Sequencing Consortium 2006). An additional 336 bp were obtained from intronic sequences between *dl* exons 4 and 5, *myd88* exons 1 and 2, *pli* exons 2 and 3, and *pro-PO* exons 8 and 9 (supplementary table S1, Supplementary Material online). DNA extraction, PCR, and sequencing reaction conditions are described elsewhere (Kent et al. 2011; Harpur et al. 2012). Briefly, we amplified each locus from each worker via PCR. Amplicons were then sequenced via two independent reactions using forward and reverse primers. Sequences have been deposited to Genbank (accession nos. KC766133–KC767183). Our group previously investigated approximately 16.5 kb of exonic sequences from 20 randomly selected and unlinked honey bee genes (Harpur et al. 2012; Kent et al. 2012). These 20 exons (supplementary table S2, Supplementary Material online) were selected using a random number generator and sequenced using PCR primers as described for the immune genes above. We amplified Toll Pathway genes using the same DNA templates as the random genes (i.e., genes were amplified from the same bees in both the random gene set and the Toll gene set). The random gene set contained 8 genes with no *Drosophila* orthologs (i.e., Hymenoptera- or Bee-specific genes) and 12 genes with *Drosophila* orthologs. Although the number of genes with *Drosophila* orthologs was too small to warrant a formal annotation enrichment analysis, the random genes did not seem enriched for any specific function based on an inspection of Gene Ontology annotation terms (Ashburner et al. 2000); there were no cases where more than three genes had the same annotation term.

Sequence Analyses

We used standard methods for detecting and validating single nucleotide polymorphisms (SNPs) from Sanger sequencing of diploid individuals (Gos and Wright 2008). Sequencing reads were imported into Sequencer (v4.10.1), trimmed of low-quality bases, and aligned to the published *Apis mellifera* genome (Kent et al. 2011; Harpur et al. 2012). Heterozygous sites were detected using Sequencer’s Call Secondary Peaks command with a 34% threshold and checked manually for errors. As PCR has very low error rates (1 x 10^-5 to 1 x 10^-6, Cline et al. 1996) and because we trimmed the sequences of low quality bases, the two independent sequencing reactions per locus per bee provide a basis for validating the detected mutations (Gos and Wright 2008). Additionally, PCR/
sequencing artifacts, if present, would have affected both the random and immune gene sets equally, and would’ve affected estimates at both silent and replacement sites. The highly nonrandom enrichment of NS mutations in Toll genes is not likely to be an artifact of PCR/sequencing errors. Because our samples were of diploid origin, sequences downstream of insertion/deletions (indels) polymorphisms could not be resolved due to shifted sequencing peaks. Although we could ascertain the sequence flanking indel polymorphisms, we could not ascertain the genotypes of individuals at indel polymorphisms. We excluded indel polymorphisms in introns in all bees from our analyses but retained SNPs in flanking intronic sequences. The SNPs analysed herein were discovered from the alignments as allelic differences within A. mellifera or between A. mellifera and A. cerana.

Population Genetic Analysis
We used Phase (Stephens and Scheet 2005) to estimate the haplotypes present in the sampled diploid workers. We used DNAsp 5.10 (Librado and Rozas 2009) to estimate several population genetic parameters and summary statistics including Tajima’s D (Tajima 1989), Fu and Li’s D* and F* (Fu and Li 1993), the number of nonsynonymous (Ka) and synonymous nucleotide (Ks) differences between A. mellifera and A. cerana, the rate of amino acid evolution (Ka/Ks), and nucleotide diversity at synonymous (πs) and nonsynonymous (πns) sites (Nei 1987). Significance of Tajima’s D and Fu and Li’s D* and F* were determined through 100,000 coalescent simulation runs using estimated recombination rates with DNAsp 5.10. We constrained the diversity and divergence observed in Toll pathway genes relative to the 95% CIs for πs, πns, Ka, Ks, and Ka/Ks observed in the random gene set. The random gene set provided an estimate of the average trends of the genome, and thus values outside the 95% CI of the random gene set were considered extreme. We estimated the earlier mentioned summary statistics using the following: 1) All A. mellifera samples treated as a single population (hereafter referred to as the “Global” A. mellifera population), or 2) only honey bees from the African subspecies A. mellifera scutellata (hereafter referred to as “African”) representing an old, large, and stable population that is minimally impacted by human management, following Kent et al. (2012).

We used the MK test for detecting selection, as implemented in DNAsp 5.10 (McDonald and Kreitman 1991). This test compares the ratio of synonymous and nonsynonymous polymorphisms within a species to fixed synonymous and nonsynonymous mutations between species: an excess of fixed: polymorphic nonsynonymous mutations relative to synonymous mutations is indicative of positive selection rapidly fixing beneficial mutations. Significance for MK tests was assessed with a Fisher’s exact test after applying a 5% FDR (Benjamini and Hochberg 1995). We estimated the proportion of mutations fixed by selection, α for each gene (Smith and Eyre-Walker 2002). Because α theoretically ranges from 0 to 1 but its estimator can take on negative or undefined values if there are few or no fixed nonsynonymous mutations, genes with negative or undefined α were set to 0. Although the MK test is largely insensitive to population demography (McDonald and Kreitman 1991), we used polymorphism data from African honey bees to ensure that MK tests are not influenced by the demographic events such as out-of-Africa expansions (Whitfield et al. 2006).

Balancing selection and reduced selective constraint can both generate an excess of nonsynonymous polymorphisms, but the former is not expected to increase divergence. We used DNAsp to perform HKA tests (Hudson et al. 1987) to compare the ratio of divergence (K) to polymorphism (π) at candidate innate immune genes relative to a presumably neutral locus (i.e., random gene set or intronic sequences). High levels of interspecific diversity relative to interspecific divergence at the target locus, relative to the neutral locus, is indicative of balancing selection acting on the former (Hudson et al. 1987). Similar to MK-tests, we only used African honey bee samples to generate polymorphism data for HKA tests. Finally, we used LOSITAN (Antao et al. 2008) to estimate global Fst and pairwise Fst for each gene between old world populations (Africa, East Europe, and West Europe) (Weir and Cockerham 1984).

We compared summary stats (e.g., diversity, divergence, and Tajima’s D), MK-α, and Fst between Toll genes and random genes using ANOVA as implemented in R v.2.11.1 (R Development Core Team 2010). We report two-tailed P values unless otherwise indicated. We used a one-tailed ANOVA to statistically compare the allele frequency spectrum of nonsynonymous mutations in all Toll-pathway genes that have nonsynonymous mutations because relaxed-selective constraint is expected to skew the frequency spectrum of nonsynonymous mutations toward intermediate (i.e., higher D at nonsynonymous sites) frequencies (Nei 1987; Tajima 1989); these tests were conducted for the Global and African population; we did not conduct this test on West European and East European samples separately because such population have low diversity and very few NS polymorphisms (Harpur et al. 2012).

We had previously identified an effect of GC content on the molecular evolution and population genetics of honey bee genes (Zayed and Whitfield 2008; Kent et al. 2012). We found no differences between the GC content at the third codon position (i.e., GC3) of Toll genes and the random gene set studied herein (F1,30 = 0.91, P = 0.35). As such, our comparisons should not on average be influenced by variation in GC content.

Protein Structure
One of the immune genes, pgrp-sa, had evidence of balancing selection. To identify functional domains of PGRP-SA, we obtained a structure of the protein from vertebrates (Sharma et al. 2008) and insects (Reiser et al. 2004). After aligning the translated sequence of pgrp-sa from the honey bee to the known protein sequences (Reiser et al. 2004; Sharma et al. 2008) using ClustalW2 (Larkin et al. 2007), we mapped SNPs and protein regions under selection onto PGRP-SA’s structure using Chimera15.2 (Pettersen et al. 2004).
Sequence similarity between honey bee PGRP-SA and the proteins with known structure was high (e value < 10^{-55}). PGRP-SA has two major protein domains, a peptidoglycan-recognition domain that is responsible for recognition of bacterial cell walls, and a PGRP-specific domain (α-helix 2 and the N-terminus: 45% of exon 3) that likely functions in protein–protein interactions and subsequent signal transduction (Sharma et al. 2008).

**Supplementary Material**

Supplementary tables S1–S4 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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