A-to-I RNA editing in honeybees shows signals of adaptation and convergent evolution

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SUMMARY

Social insects exhibit extensive phenotypic diversities among the genetically similar individuals, suggesting a role for the epigenetic regulations beyond the genome level. The ADAR-mediated adenosine-to-inosine (A-to-I) RNA editing, an evolutionarily conserved mechanism, facilitates adaptive evolution by expanding proteomic diversities. Here, we characterize the A-to-I RNA editome of honeybees (*Apis mellifera*), identifying 407 high-confidence A-to-I editing sites. Editing is most abundant in the heads, and shows signatures for positive selection. Editing behavior differs between foragers and nurses, suggesting a role for editing in caste differentiation. Although only five sites are conserved between bees and flies, an unexpectedly large number of genes exhibit editing in both species, albeit at different locations, including the nonsynonymous auto-editing of *Adar*. This convergent evolution, where the same target genes independently acquire recoding events in distant diverged clades, together with the signals of adaptation observed in honeybees alone, further supports the notion of recoding being adaptive.

Keywords: A-to-I RNA editing; Honeybee; Caste-dependent editing; Adaptation; Convergent evolution.

INTRODUCTION

Adenosine (A) to inosine (A-to-I) RNA editing, catalyzed by enzymes of the ADAR (adenosine deaminase acting on RNA) family (Savva et al. 2012b), is an evolutionarily conserved mechanism that expands RNA diversity at the co-transcriptional or post-transcriptional level in metazoans (Bass 2002; Nishikura 2010; Eisenberg and Levanon 2018). Two catalytically-active *Adar* genes are encoded in most metazoans. However, insects have lost ADAR1 and encode only a single *Adar* gene (Keegan et al. 2011). Due to the structural similarity between inosine (I) and guanosine (G), I is generally believed to be recognized as G in many cellular processes such as mRNA splicing (Rueter et al. 1999; Flomen et al. 2004; Jin et al. 2007; Lev-Maor et al. 2007), microRNA (miRNA) biogenesis or target recognition (Liang and Landweber 2007; Borchert et al. 2009; Alon et al. 2012), and mRNA translation (Basilio et al. 1962; Licht et al. 2019). Therefore, an A-to-I RNA editing usually has a similar effect as an A-to-G DNA substitution. A-to-I editing plays essential roles in many biological and physiological processes(Keegan et al. 2001; Nishikura 2010), and dysregulation of A-to-I editing might be associated with cancer, autoimmune disorders, or other human diseases (Gallo et al. 2017).

During the past two decades A-to-I RNA editing sites have been systematically characterized in various metazoan species (Ramaswami and Li 2016). The majority of RNA editing sites are located in clusters in non-coding regions of humans (Athanasiadis et al. 2004; Blow et al. 2004; Kim et al. 2004; Levanon et al. 2004; Picardi et al. 2017), monkeys (Chen et al. 2014; Yang et al. 2015), mice (Neeman et al. 2006; Danecek et al. 2012), worms (Morse and Bass 1999; Zhao et al. 2015; Goldstein et al. 2017), corals (Porath et al. 2017b) and many other species (Porath et al. 2017a). In most species, only a minute fraction of the edits resides within the coding sequence. Notable exceptions are *Drosophila* (Graveley et al. 2011; Rodriguez et al. 2012; St Laurent et al. 2013; Mazloomian and Meyer 2015; Yu et al. 2016; Buchumenski et al. 2017; Duan et al. 2017; Zhang et al. 2017) and cephalopod species (Alon et al. 2015; Liscovitch-Brauer et al. 2017). Despite the deep conservation of A-to-I editing mechanism, the target landscapes of editing have considerably evolved during metazoan evolution. Only one editing site is known to be conserved across virtually all mammals, *Drosophila*, and cephalopods (Porath et al. 2019), and it thus seems that RNA editing modulates the diversity of

92 the transcriptomes and proteomes in a lineage-specific manner.

93 The forces driving the evolution of A-to-I editing across species at the macro-evolutional
94 scale are not well understood. RNA editing was hypothesized to facilitate adaptive evolution
95 by increasing proteomic diversities temporally or spatially, in a manner more flexible than
96 genomic mutations (Gommans et al. 2009; Nishikura 2010; Klironomos et al. 2013; Rosenthal
97 2015; Nishikura 2016). However, in most species studied the ratio of nonsynonymous *(N)* to
98 synonymous (*S*) editing sites (*N/S*) is lower than expected for random sites, suggesting that
99 recoding events may be overall non-adaptive (Xu and Zhang 2014). Here too *Drosophila* and
100 cephalopods stand out as exceptions, exhibiting high *N/S* ratios indicating positive selection of
101 recoding. Furthermore, hundreds of recoding sites were shown to be conserved across the
102 *Drosophila* lineage (Graveley et al. 2011; Rodriguez et al. 2012; St Laurent et al. 2013;
103 Mazloomian and Meyer 2015; Yu et al. 2016; Buchumenski et al. 2017; Duan et al. 2017; Zhang
104 et al. 2017), and thousands in the behaviorally sophisticated cephalopods (Garrett and
105 Rosenthal 2012; Alon et al. 2015; Liscovitch-Brauer et al. 2017), supporting the functional
106 importance of these editing events. However, in only a few examples was the advantageous
107 effect conferred by RNA editing explicitly demonstrated (see for example (Garrett and
108 Rosenthal 2012)), and even it is not yet clear to what extent editing is indeed utilized for
109 proteome diversification.

110 Social insects, including bees and ants, show extensive phenotypic plasticity. The
111 morphologically and behaviorally differentiated social castes such as queens, workers, and
112 drones have the same set of diploid or haploid genome. The social insects provide us with model
113 system to study how phenotypic diversity is regulated (Page et al. 2012; Yan et al. 2014). RNA
114 editing is well suited to contribute to the behavioral variation among genetically similar
115 individuals. Indeed, differential editing was demonstrated for a few sites in leaf-cutting ants
116 and in worker bumblebees (Li et al. 2014; Porath et al. 2019).

117 Here we wish to study the contribution of RNA editing to caste differentiation in honeybee
118 (*Apis mellifera*), an important pollinator and a model for complicated social behaviors in insects
119 (Page et al. 2012). Unlike bumblebees, honeybees have a sharp task-specialization, with distinct
120 worker castes. Genetic mapping demonstrated that the phenotypic plasticity in honeybees is
121 associated with complex epistatic and pleiotropic genetic networks that influence reproductive

122 regulation and foraging behaviors (Page et al. 2012). We wish to check whether A-to-I RNA
123 editing may contribute to the proteomic diversity underlying this phenotypic plasticity. We
124 investigate the A-to-I RNA editome in different tissues of four honeybee drone individuals and
125 detect over four hundred A-to-I sites in multiple tissues. Editing is enriched in the head, and
126 exhibit signs for positive selection and a particularly high *N/S* ratio. We show editing is elevated
127 in foragers compared to nurses. Five editing sites are conserved between honeybee, bumblebee
128 and *Drosophila*. Interestingly, we find a significantly high number of cases where the same
129 gene in edited in both bees and flies, even if not at the same position. One example is the auto-
130 editing of *Adar* mRNA in bees and flies, which might play an auto-regulatory role in the two
131 clades. This finding may further support possible convergent evolution and adaptation.
132

133 **RESULTS**

134 **Editome of honeybee**

135 We deep-sequenced genomic DNA and RNA from the head, thorax, and abdomen for each
136 of four individual drones (12 RNA-seq samples) (Figure 1A). The transcriptome libraries of
137 drones 1 and 2 were constructed by selecting polyA tailed mRNAs while the libraries of drones
138 3 and 4 were constructed by using the Ribo-Zero kit to deplete ribosomal RNAs (Transparent
139 Methods). The male drone is haploid, simplifying identification of its genomic SNPs. DNA-
140 Seq reads were mapped to the reference genome to identify 826,890-858,949 SNPs in each of
141 the four drones, and 1,303,225 unique sites combined (Transparent Methods; Table S1). Median
142 sequencing coverage at the SNP sites was 39, and in 96.6% of the sites all DNA reads supported
143 the SNP (Supplemental Fig S1). The identified SNPs were then used to produce a masked
144 genome version where the reference allele is substituted by the individual-specific alleles
145 identified for each drone, to facilitate accurate detection of RNA editing sites (Figure 1B;
146 Transparent Methods).

147 For each of the 12 RNA-Seq samples, 9.2-21.5 million reads were uniquely mapped to the
148 reference genome, and similar numbers of reads were mapped to the masked genome sequences
149 (Transparent Methods; Table S2). Analyzing pooled data for each of the three tissues separately,
150 we identified (FDR=0.05) 376 (84.3%) A-to-G sites among 446 variations in heads, 106 (66.7%)
151 A-to-G sites among 159 variations in thoraxes, and 137 (67.5%) A-to-G sites among 203

152 variations in abdomens (Figure S2). Combined, we obtained 407 (80.1%) unique A-to-G sites
153 among 508 variations (Figure 1C). These 407 sites are regarded as A-to-I RNA editing sites
154 (Table S3, Information of the candidate editing sites identified in this study, Related to Figure
155 1). The nucleotide context around these putative editing sites is consistent with the known
156 ADAR binding motif (Figure S3). The median DNA coverage (over the 407 A-to-I editing sites)
157 was 25 reads, sufficient to exclude possible SNPs (Figure S4).

158 To improve mapping accuracy, we discarded variants in repeat regions, and required a
159 variation site to be found by mapping to both the reference genome and the masked genome
160 (Transparent Methods). To test whether these criteria are too stringent, we checked the variation
161 sites discarded by the two filtering steps. First, we looked at the variation sites located in the
162 repeat regions. Without any filter, we found 19,573 unique variation sites that overlapped with
163 repeat regions, only 3,694 (19%) of which were A-to-G variants. Following a binomial test and
164 multiple testing correction (to remove random sequencing errors), 1,277 variation sites were
165 maintained, only 324 (25%) of them A-to-G variations. Thus, filtering repetitive regions does
166 contribute appreciably to the precision of our detection. Consistently, hyper-editing analysis did
167 not reveal multiple sites in repetitive regions (Transparent Methods).

168 As for the masked genome filter, only 38% (98/256) of the variation sites found by
169 mapping to the masked genome but not to the reference genome are A-to-G mismatches.
170 Following binomial test for sequencing error and multiple testing correction for *P* values, only
171 41 variation sites were maintained, none of them was an A-to-G variation. Conversely, only 31%
172 (82/268) variation sites found by mapping to the reference genome but not to the masked
173 genome are A-to-G mismatches. Of these 268 variation sites, 82 were maintained after multiple
174 testing correction, 20 of them were A-to-G variations. Thus, most of the sites supported by only
175 one method of mapping are likely not due to A-to-I RNA editing.

176 Among the 407 editing sites we identified, 199 sites are located in gene regions, including
177 111 nonsynonymous (*N*) sites, 9 synonymous (*S*) sites, and several sites in UTRs and introns
178 (Figure 1D). The other 208 sites are annotated as intergenic. As expected from the different
179 library construction strategies, the fraction of sites in coding regions is higher and the intergenic
180 fraction is lower for sites observed in drones 1 and 2 compared with drones 3 and 4 (23.2 ± 2.6%
181 in CDS in drones 1 and 2 versus 14.5 ± 5.1% in CDS in drones 3 and 4; 54.9 ± 2.2% in intergenic

182 regions in drones 1 and 2 versus 70.5 ± 5.4% in intergenic regions in drones 3 and 4). The
183 number of sites for which editing is observed in each sample (per-sample editing level > 0)
184 varies considerably (40-278 sites per sample; Table S4). The partial overlap between these sets
185 of sites is mostly due to sites being undetected at a given sample due to low per-sample coverage
186 (Figure S5).

187 Interestingly, editing seems to be enriched in heads. The number of detected sites (Figure
188 S6), the editing levels (Figure 1E) and the fraction of coding sites (Figure S7), are all higher in
189 heads. Clustering the samples by their editing profile, the three tissue-types form distinct
190 clusters (Figure 1F). Consistently, *Adar* expression is also higher in heads than other tissues
191 (Figure S8). Looking at specific sites, 78 sites (out of 378 sites with sufficient coverage, see
192 Transparent Methods) are differentially-edited in the head, compared to non-head tissues. In all
193 of these 78 sites, editing is higher in the head (Table S3, Information of the candidate editing
194 sites identified in this study, Related to Figure 1).

195

196 **Signals of adaptation**

197 The nonsynonymous to synonymous (*N*/*S*) ratio for sites in the coding region is 111/9 = 12.3
198 (Table S4), compared to a ratio of 2.26 observed for random adenosine to guanosine
199 substitutions (see Transparent Methods). This strongly suggests that the nonsynonymous editing
200 events in honeybees are overall adaptive. Notably, the *N*/*S* is exceptionally high, even compared
201 to the ratios we previously found in the brains of *Drosophila* (Duan et al. 2017) or in
202 cephalopods (Alon et al. 2015; Liscovitch-Brauer et al. 2017). Looking at each sample separately,
203 at pooled tissue data, or focusing on sites that appear in at least two individuals per tissue all
204 show the *N*/*S* ratio to be especially high in heads (Figure 2A and Table S4). Furthermore, the
205 editing level at nonsynonymous sites is significantly higher than synonymous sites editing
206 levels in heads of honeybees (Figure 2B).

207 A previous study in cephalopods proposed that the coleoids massively edit their RNAs to
208 diversify the transcriptome at the cost of constraining the evolution of genomic sequences
209 (Liscovitch-Brauer et al. 2017). Maintaining beneficial editing requires genomic conservation
210 of the genomic sequence encoding for the dsRNA structures that allow ADAR to bind and
211 deaminate the adenosine. To test this, we first confirmed that the editing sites in honeybees are

212 enriched in hairpin structures (Figure 2C). Then, we demonstrated that SNPs are depleted in
213 the vicinity of nonsynonymous sites (Figure 2D), further supporting the notion of selective
214 advantage for the nonsynonymous editing events that justifies the tradeoff between
215 transcriptome diversity and genome evolution.
216
217 **Evolutionarily conserved and non-conserved editing sites**
218 Editing at four nonsynonymous and one synonymous site is conserved between
219 *Drosophila* and honeybees (Figure 3). Three of these sites are in the *Shab* transcript (*shaker*
220 *cognate b*, two nonsynonymous and one synonymous) and the other two are is in *qvr* (*quiver*).
221 Editing at these sites is also observed in the bumblebee *Bombus terrestris* (Porath et al. 2019),
222 and editing levels are high in all of these species (Figure 3). These findings suggest a potential
223 functional role for these widely conserved editing sites. However, the majority of the editing
224 sites are not shared between *Drosophila* and honeybees. For some sites the editable adenosine
225 is not conserved in the honeybee genome (Figure S9), and are thus clearly uneditable, while
226 other adenosines are conserved but editing was not detected in RNA-Seq data (Figure S9).
227 Overall, of the 639 *Drosophila* editing sites in CDS for which an orthologous site was identified
228 in honeybees, 349 were conserved as A (55%), and 85, 127, and 78 (13%, 20%, and 12%) were
229 mutated to C, G and T, respectively.
230 Similarly, most sites are not even conserved across the two bee species. The bumblebee
231 study has reported 219 editing sites in CDS, 164 of which are nonsynonymous (Porath et al.
232 2019). Of these, we found 9 editing sites in coding regions conserved between honeybee and
233 bumblebee (Table S5).
234 One of these conserved sites is a Ser>Gly site within the *tipE* (temperature-induced
235 paralytic E; *GB47375*) transcript, editing levels of which correlate with task-performance in
236 bumblebees (nurses versus foragers) (Porath et al. 2019). Editing level at this site in honeybee
237 heads are ~0.7, similar to those observed in bumblebee brains. In the two other tissues this gene
238 is lowly expressed and poorly edited. The orthologous site in *Drosophila* genomes encodes the
239 post-edit Gly codon GGC (Figure S10). Thus, *tipE* recoding seems to be bee-specific, possibly
240 related to social behavior and task performance.
241

242 **Sex-dependent and Caste-dependent editing**

243 We further looked for potential sex-dependence and caste-dependent editing, comparing
244 our honeybee drone head data with previously published brain RNA-seq data studying two sub-
245 castes of female workers, foragers and reverted nurses (Herb et al. 2012) (Table S6). First, we
246 compared the pooled editing levels between drones and workers for each well-covered (>10
247 reads in each pool) editing site separately. Twenty six sites exhibit differential editing
248 (Wilcoxon rank sum test; FDR=0.05) between workers (females) and drones (males), including
249 4 recoding sites, 2 sites in 3'UTRs, 3 intronic sites, and 17 intergenic sites (Figure 4A and Table
250 S7, Differentially edited sites between drones and workers, Related to Figure 4). In most cases
251 editing in drones is higher, but this may result from looking at sites identified in drone data to
252 begin with. The difference in *Adar* expression was not statistically significant (Figure S11).

253 Comparing the pooled editing levels between the two sub-castes (foragers and nurses), one
254 finds 230 out of 407 sites with lower levels in nurses, compared to only 91 exhibiting higher
255 levels in nurses (equal level, mostly zero, was observed in 84 sites), suggesting a globally higher
256 editing activity (at the detected sites) in foragers (P = 1.3e-14, proportion test). The surcharge
257 of forager-higher sites is maintained for various coverage and editing-difference cutoffs (Figure
258 S12). Furthermore, *Adar* expression (Figure 4B) and the editing index (Figure 4C) are both
259 higher in foragers, and the editing index correlates with *Adar* expression. However, reliable
260 detection of specific differentially-edited sites with the available sample size (6 foragers and 6
261 nurses) is challenging, and not even a single site was identified with FDR=0.05 (Wilcoxon rank
262 sum test). We have thus relaxed the statistical test, allowing for FDR=0.3, and found 61
263 candidate sites, most of which are differentially edited (Figure 4D and Table S8, Differentially
264 edited sites between foragers and nurses, Related to Figure 4). These include 10
265 nonsynonymous, 16 3'UTR, and 35 intergenic sites. Consistently, 55 of these sites exhibit
266 higher levels of editing in foragers. Finally, PCA analysis of the editing profile across sites
267 results in distinct clusters of drones, nurses and foragers, a classification that is not achieved by
268 the expression profile (Figure 4E and 4F). Taken together, these results show a global increase
269 in editing in foragers compared to nurses.

270

271 **Convergent adaptation of A-to-I editing?**

One of the recoding sites conserved between the two bee species resides within *Adar* transcript (Table S5 and Figure 5A). In bumblebee, the recoding level of the conserved site, I482M (ATA to ATG), positively correlates with the global editing activity (Porath et al. 2019), suggesting a possible auto-regulation mechanism. Interestingly, *Drosophila Adar* is also auto-edited at a different position, where a Ser (AGT) to Gly (GGT) substitution leads to a less active ADAR protein, resulting in a negative feedback loop of editing activity (Palladino et al. 2000; Savva et al. 2012a). Intriguingly, fly-edited Ser amino-acid is conserved in honeybee and bumblebee, but a different codon (TCA, uneditable at the first codon position) is used. Similarly, the bee-edited Ile is conserved in flies, but the edited adenosines (at the third position of ATA) is synonymously mutated to T, and the editable ATA bee codon is substituted by ATT codon in flies, which could not be edited at the third position (Figure 5A). Thus, while auto-editing S430G in flies is abolished in bees due to an uneditable Ser codon, and the auto-editing I482M in bees is abolished in flies due to an uneditable Ile codon. However, the mechanism of auto-editing, possibly used for global ADAR regulation, is shared by the two lineages, albeit at different positions and possibly with different effects on the protein. This might hint at the possibility of convergent evolution of ADAR auto-regulation strategy.

Following this example, we wondered whether there are more genes for which editing is observed in both honeybees and *Drosophila*, even if the exact location of the editing site is not conserved. Excluding conserved sites, there are 53 genes exhibiting CDS editing in honeybee heads, 101 genes exhibiting CDS editing in bumblebee heads, and 312 genes edited in CDS in male brains of *D. melanogaster*. Of these, 21 genes exhibit editing in both bee species, 14 are edited in honeybee and *Drosophila,* 14 are edited in bumblebee and *Drosophila*, and six genes are found to be edited in all three species (Figure 5B and Table S9). These numbers are significantly higher than expected by random sampling, ($p<1e-6$, randomization test), even if one accounts for the expression profile (Transparent Methods, Figure 5C and Figure S13). Table S9). We have repeated the analysis using stricter criteria to characterize sites, considering as edited only sites with an observed editing level >10% (all samples pooled), and a binomial $P < 0.05$ (excluding the null hypothesis of no editing), and considering a site to be not-edited if the binomial $P < 0.05$ for the null hypothesis that the site is edited at 10%. Reassuringly, the results are robust to this change of definitions (Figure S14 and S15). These results suggest an

302 interesting convergent evolution of A-to-I editing. Recoding sites are rarely conserved across
303 clades, but the same genes in different lineages tend to acquire a recoding event, apparently
304 independently, indicating a functional importance for recoding of this gene.
305

306 **DISCUSSION**

307 Despite over fifteen years of developments, reliable detection of CDS editing remains a
308 challenge. Systematic searches often lead to high false-positive rates (reflected by the fraction of
309 non-AG mismatches observed), especially for mammalian transcriptomes where the scope of
310 recoding is rather low. Important exception are *Drosophila* and cephalopods, where the recoding
311 signal is much more pronounced and easier to detect. Applying a novel strict alignment approach,
312 we were able to achieve here a high accuracy in CDS editing detection (84.3% in heads) despite the
313 overall modest scope of recoding sites. Another similarity between honeybees *Drosophila* and
314 cephalopods is related to the important question regarding the extent to which recoding is adaptive.
315 This is often estimated by the *N/S* ratio, where *Drosophila* and cephalopods exhibit a pattern
316 markedly different from the one seen in other species studied, (including human). Whereas in most
317 species the *N/S* ratio observed is lower or similar to the one expected under neutrality, it is
318 significantly higher in *Drosophila* and cephalopods. Again, we find here that honeybees show a
319 strikingly high *N/S* ratio, similar to the above two clades. We suggest that the two last points are
320 interconnected. The apparently low *N/S* ratio observed in many species (human included) may
321 reflect the low accuracy of the lists of CDS editing sites and not the (lack of) adaptive potential for
322 recoding in these species.

323 The importance of post-transcriptional and post-translational mechanisms in generating the
324 proteomic complexity of higher organisms has been emphasized in recent decades. These epigenetic
325 mechanisms allow for diversification of the proteome and functional heterogeneity across tissues,
326 developmental stages, brain regions or even among individual cells.

327 Recoding by A-to-I RNA editing is an epigenetic mechanism capable of diversifying the
328 proteome, creating a range of proteins from a single genomically encoded gene in a temporally-
329 regulated, tissue-specific, condition-dependent way, and providing the organism with a new means
330 for acclimation and adaptation. Indeed, several studies have demonstrated how recoding levels at
331 specific sites do change as a function of the organism's condition (Garrett and Rosenthal 2012;

332 Robinson et al. 2016; Gallo et al. 2017; Porath 2017; Terajima et al. 2017; Yablonovitch et al.
333 2017). Importantly, many studies have demonstrated altered editing of individual recoding targets
334 in various disease states (Gallo et al. 2017). However, these interesting examples notwithstanding,
335 the extent to which the recoding phenomenon is actually used as a means for proteome
336 diversification is still under debate. In fact, several recent studies have raised the possibility that
337 nonsynonymous editing may be used to compensate for otherwise deleterious G-to-A mutations,
338 rather than allowing for the two alleles (A and G) to co-exist (Jiang and Zhang 2019; Mai and
339 Chuang 2019; Popitsch et al. 2020). Thus, a high *N*/*S* ratio is not sufficient to prove that editing
340 serves for adaptation through proteome diversification.

341 The behavioral, physiological and morphological plasticity exhibited by social insects
342 suggests an important role for epigenetic mechanisms. Differential RNA editing was previously
343 shown for different castes of the highly social leaf-cutting ant *Acromyrmex echinatior* (Li et al.
344 2014), as well as for bumblebee workers (Porath et al. 2019). Our results expand these finding
345 to another species, further supporting the notion of RNA editing as a source of proteomic
346 complexity, that may be recruited to provide phenotypic variation among genetically identical
347 individuals.

348 ADAR enzymes, and the recoding phenomenon are widely conserved across metazoan.
349 However, the repertoire of recoding sites varies considerably and seems to have developed almost
350 independently in different clades. Here we point out that the same gene targets are being edited in
351 distant species, even if the exact locations of the editing sites within the transcript vary. A
352 particularly interesting example is that of nonsynonymous auto-editing of *Adar*, shown to affect
353 ADAR activity in bumblebees, flies and even mammals (Rueter et al. 1999; Savva et al. 2012a;
354 Porath et al. 2019), possibly serving as global editing autoregulation. It thus seems possible that
355 the selective advantage in having multiple versions of the protein product is shared by the target
356 genes, while there is more than one specific way to achieve this diversity through recoding. Clades
357 developed along different evolutionary routes have converged on different implementations (in
358 terms of the specific editing site) of the same diversifying solution. These ideas should be further
359 tested in the future, using larger datasets and across additional species and clades. Moreover, as
360 biochemical and functional understanding of the impact of the different edits are gained, it will be
361 possible to look into a possible common effect for the different edits of the common targets.

362

363 **Limitations of the Study**

364 The editomes of the bee species analyzed here are based on a limited number of samples. They
365 probably represent a subset of the actual repertoire of editing sites. The results should be re-tested
366 when more data is available. In particular, the honeybee editome was built using drone samples.
367 Thus, some individual differential editing between the nurses and foragers sub-castes may have
368 missed worker-specific sites. However, the global increased editing in foragers, supported by a
369 higher Adar expression, is probably robust.

370

371 **Resource Availability**

372 *Lead Contact*

373 Further information and requests for resources should be directly and will be fulfilled by the
374 Lead Contact, Jian Lu (luj@pku.edu.cn).

375 *Materials Availability*

376 This study did not generate new unique materials.

377 *Data and Code Availability*

378 All deep-sequencing data generated in this study were deposited in the China National
379 Genomics Data Center Genome Sequence Archive (GSA) under accession number
380 CRA002262. Other deep-sequencing data analyzed were downloaded from SRA as follows.
381 Brains of honeybee workers: accession numbers SRR445999 to SRR446004 (reverted nurse)
382 and SRR446005 to SRR446010 (forager) (Herb et al. 2012). Bumblebee: SRP166322 (Porath
383 et al. 2019). *D. mel* head: SRP067542 (Zhang et al. 2018). *D. sim* head: SRP074828 (Duan et
384 al. 2017). *D. pse*: DRR055250 and DRR055251 (Nozawa et al. 2016). *D. wil*: SRR341127 and
385 SRR341129 (Meisel et al. 2012). *D. moj*: SRR037508-SRR037518 (generated by the
386 *Drosophila* modENCODE project). *D. sec*, and *D. ere* data are unpublished data generated in
387 our own lab. The reference genomes versions used for *Drosophila* species are Dmel_r6.04
388 (http://flybase.org/) for *D. mel*, and versions droSim1, droSec1, droEre1, dp4, droWil1, and
389 droMoj3 downloaded from UCSC Genome Browser (http://genome.ucsc.edu/) for the other fly
390 species. For the *Bombus terrestris*, we used version Bter_1.0.

391

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

ACKNOWLEDGMENTS

We thank the National Center for Protein Sciences at Peking University for technical assistance. Part of the analysis was performed on the High Performance Computing Platform of the Center for Life Science at Peking University. This work was supported by the joint NSFC-ISF program (NSFC grant number 3201101147 to J.L. and ISF grant number 3371/20 to E.E.), as well as grants from the Ministry of Science and Technology of the People's Republic of China (2016YFA0500800) and National Natural Science Foundation of China (91731301) to J.L. and support from the Israel Science Foundation (1945/18) to E.E.

AUTHOR CONTRIBUTIONS

J.L. designed the research; J.X.H. contributed the honeybee materials; S.Q.D. performed the research; Y.G.D., H.T.P, E.E. and J.L. performed the bioinformatics analyses; E.E. and J.L. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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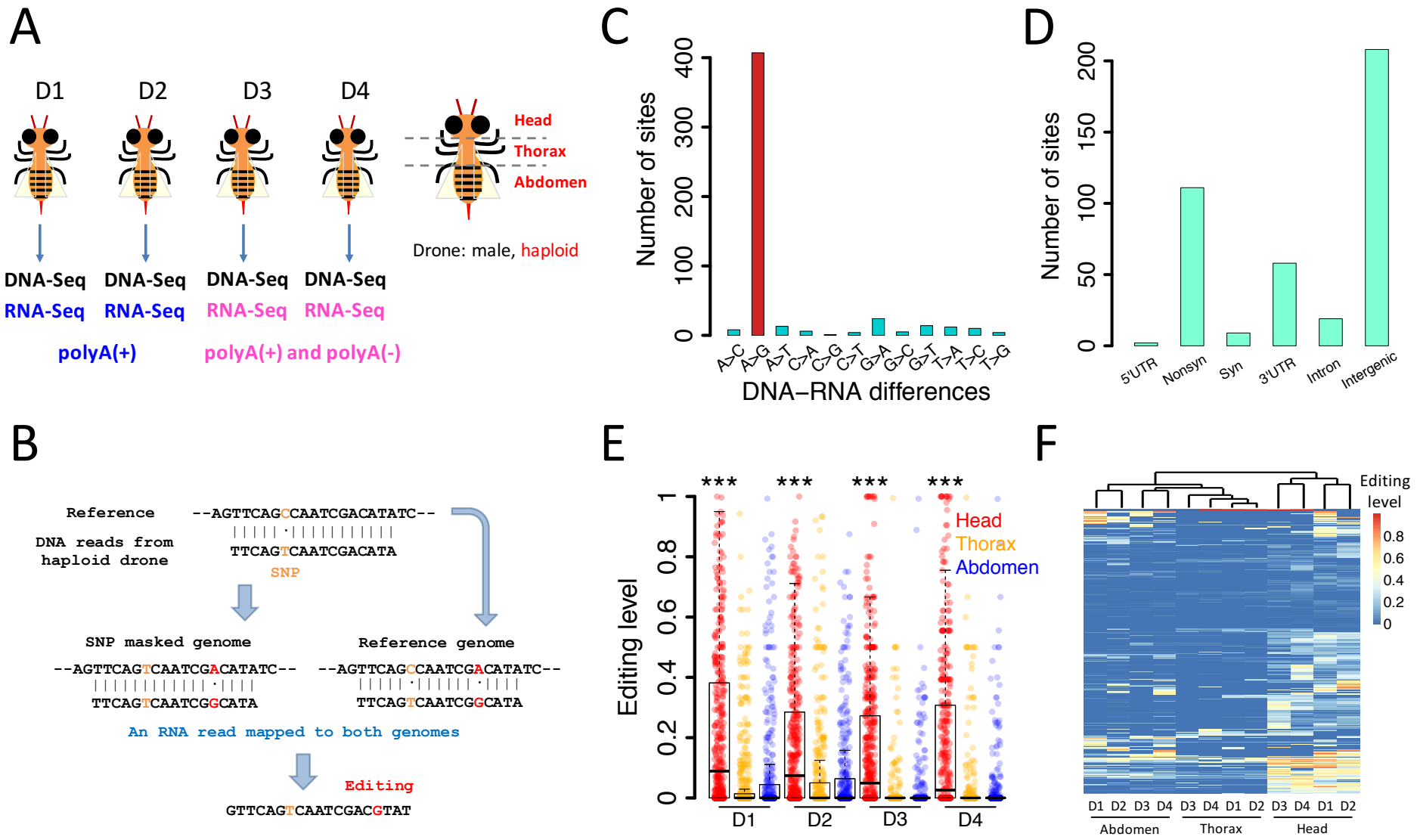
556
557
558 **Figure legends**
559 **Figure 1. Identification and annotation of the A-to-I RNA editing sites in honeybee.** (A)
560 Workflow of sample collection, dissection and library construction. (B) Identification of A-to-
561 I RNA editing sites is facilitated by mapping to both the reference genome and the masked
562 genome. (C) Distribution of DNA-RNA mismatch types detected in honeybee. (D) Distribution
563 of the detected A-to-I editing sites over different genic regions. (E) Editing levels at the detected
564 as measured in three tissues of each of four individuals (D1-D4). Editing levels are higher in
565 the head (head versus pooled thorax and abdomen, Wilcoxon rank sum test; ***: $P < 0.001$.
566 Exact P values, left to right: 2.1e-35, 5.7e-20, 4.0e-29, and 3.9e-38). (F) The editing profiles
567 for the twelve tissues cluster according to their tissue of origin.
568
569 **Figure 2. Signals of adaptation of A-to-I editing in honeybee.** (A) The observed numbers of
570 nonsynonymous (N) and synonymous (S) editing sites in each tissue. P values are obtained from
571 Fisher's exact test, comparing the observed N and S counts to all adenosines in the coding
572 sequence of in *A. mel* (N=4,492,737 sites where A-to-G substitution is nonsynonymous, and
573 S=1,986,130 for synonymous). "All sites" represents the numbers of sites that appear in at least
574 one individual. Comparisons for sites appearing in at least two individuals are also shown. (B)
575 Editing levels of the nonsynonymous and synonymous sites in head. Data from four drones was
576 pooled to increase the statistical power. P values are calculated using Wilcoxon rank sum test.
577 (C) The fraction of sites in predicted stable hairpin structures. The observed editing sites were
578 compared to the unedited adenosines (P-values using Fisher's exact test). (D) The SNP density

579 in the vicinity of nonsynonymous editing sites (green), other editing sites (cyan) or unedited
580 adenosines (grey).
581
582 **Figure 3. Editing sites conserved between bees and flies.** (A) One synonymous and two
583 nonsynonymous editing sites in *Shab* transcripts are edited in heads of two bee and four
584 *Drosophila* species. (B) Two nonsynonymous editing sites in *qvr* transcripts are edited in heads
585 of two bee and four *Drosophila* species. The editing sites are colored red. Editing levels
586 measured in male heads of flies (unpublished), pooled heads of honeybee drones, and brains of
587 bumblebees (Porath et al. 2019) are shown. *D. mel*, *Drosophila melanogaster*; *D. sim*,
588 *Drosophila simulans*; *D. sec*, *Drosophila sechellia*; *D. ere*, *Drosophila erecta; D. pse,*
589 *Drosophila pseudoobscura; D. wil, Drosophila willistoni; D. moj, Drosophila mojavensis; A.*
590 *mel*, *Apis mellifera*; *B. ter*, *Bombus terrestris*.
591
592 **Figure 4. Sex-dependent and caste-dependent editing.** (A) Comparison of editing levels
593 between drones (males) and workers (females). Statistically significant sites (FDR < 0.05) are
594 colored red. (B) *Adar* expression level (reads count normalized by DESeq2) in nurses and
595 foragers. Data are presented as mean ± SEM (standard error of mean). *P* value was calculated
596 using Wilcoxon rank sum test. (C) Spearman's correlation between *Adar* expression and editing
597 index (sum over all G alleles in all sites divided by sum of all the coverages in all sites) for all
598 the nurse (orange) and forager (purple) samples. (D) Comparison of editing levels between two
599 sub-castes of workers: nurses and foragers. Statistically significant sites (FDR < 0.3; see Results)
600 are colored red. (E) PCA analysis of the editing profile shows a different behavior for nurses
601 and foragers, while (F) PCA of the expression profile (genes with RPKM>1) does not lead to a
602 clear separation.
603
604 **Figure 5. Convergent evolution of editing.** (A) *Adar* transcripts are auto-edited in *Drosophila*
605 and bees. The Ser>Gly site is highly conserved across *Drosophila* species. The *Drosophila*-
606 editable Ser codon AGT is changed to an uneditable Ser codon TCA in bees. On the other hand,
607 the Ile>Met auto-editing site is conserved between honeybee and bumblebee. The bee-editable
608 Ile codon ATA (edited at the third position) appears as an uneditable Ile codon ATT in flies.

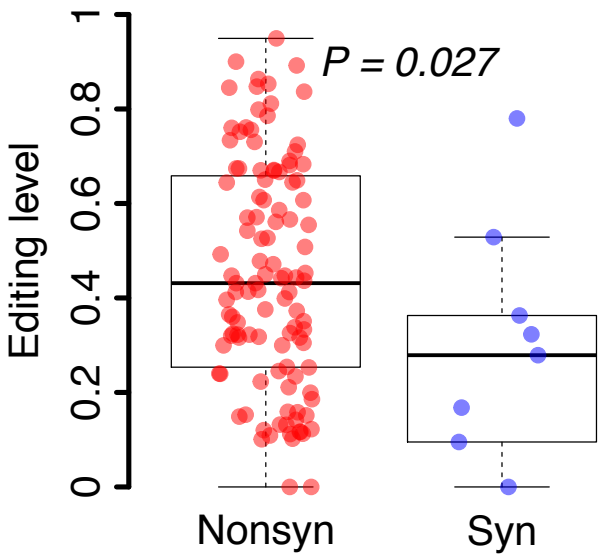
Editing sites are colored red. The editing levels from male heads of flies (unpublished), pooled heads of honeybee drones and brains of bumblebee (Porath et al. 2019) are shown. *D. mel*, *Drosophila melanogaster*; *D. sim*, *Drosophila simulans*; *D. sec*, *Drosophila sechellia*; *D. ere*, *Drosophila erecta*; *A. mel*, *Apis mellifera*; *B. ter*, *Bombus terrestris*. (B) Venn diagram demonstrating the overlap between orthologous genes with editing sites in coding regions. (C) The observed number of orthologous genes with editing in coding regions, compared to the expected distribution. Genes with RPKM > 1 in both species were chosen to calculate the expected numbers (results are essentially the same for other expression cutoffs, Figure S13). *P*-values calculated by randomization test. (D) The nonsynonymous to synonymous (*N*/*S*) ratios are significantly higher than expected, for both shared and species-specific sites (except for species-specific sites in *B. terrestris*). Here the shared and species-specific sites refer to the sites in shared genes and species-specific genes as defined in the above Venn diagram in (B). The expected ratio is evaluated by calculating the effect of putative editing on all adenosines in the coding region. *P*-values by Fisher exact test.


A
D1
D2
D3
D4
Head
Thorax
Abdomen
Drone: male, haploid
DNA-Seq
RNA-Seq
polyA(+)
polyA(+) and polyA(-)
B
Reference
--AGTTCAGCCAATCGACATATC--
DNA reads from haploid drone
TTCAGTCAATCGACATA
SNP
SNP masked genome
--AGTTCAGTCAATCGACATATC--
TTCAGTCAATCGGCATA
Reference genome
--AGTTCAGCCAATCGACATATC--
TTCAGTCAATCGGCATA
An RNA read mapped to both genomes
Editing
GTTCAGTCAATCGACGTAT
C
Number of sites
0
100
200
300
400
A>C
A>G
A>T
C>A
C>G
C>T
G>A
G>C
G>T
T>A
T>C
T>G
DNA–RNA differences
D
Number of sites
0
50
100
150
200
5'UTR
Nonsyn
Syn
3'UTR
Intron
Intergenic
E
Editing level
0
0.2
0.4
0.6
0.8
1
***
Head
Thorax
Abdomen
D1
D2
D3
D4
F
Editing level
0.8
0.6
0.4
0.2
0
D1 D2 D3 D4 D3 D4 D1 D2 D3 D4 D1 D2
Abdomen
Thorax
Head

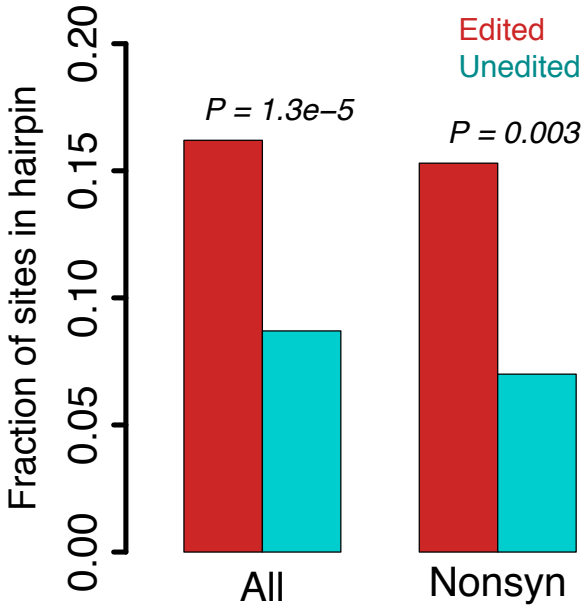
A

	N	*S*	*N/S*	*P* value
All sites				
Head	108	8	13.5	8.5E-10
Thorax	14	5	2.8	0.81
Abdomen	16	1	16	0.032
In ≥ 2 individuals				
Head	80	8	10	2.2E-6
Thorax	11	4	2.75	1
Abdomen	13	1	13	0.078

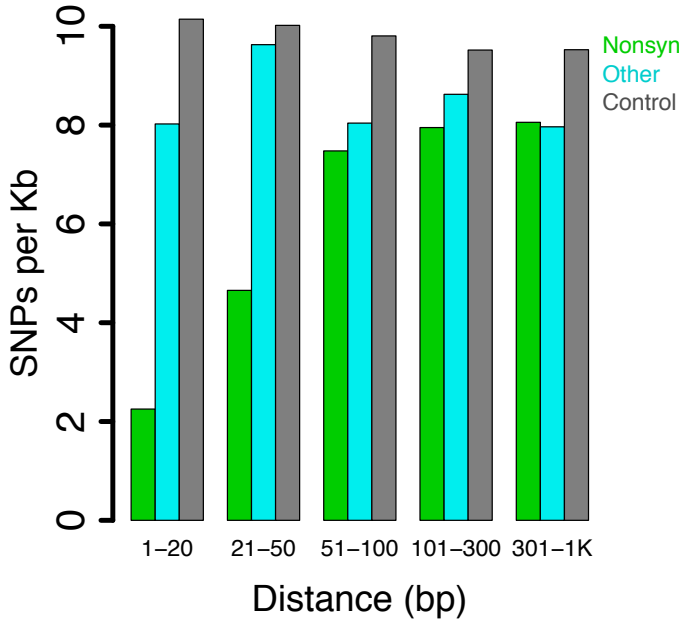
B



C



D



A

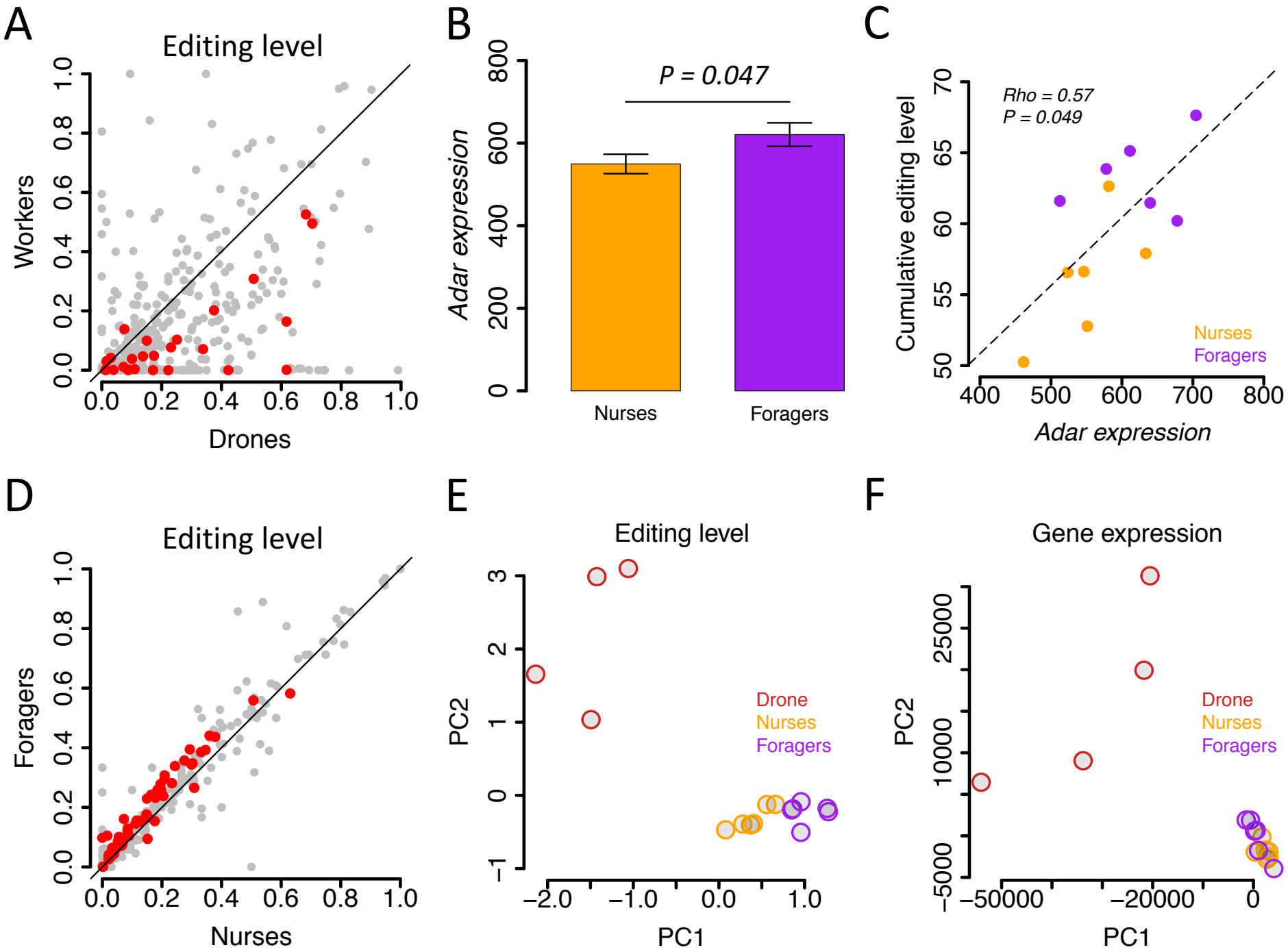
***Shab* (*shaker cognate b*)**

	Tyr>Cys				Val	Ile>Val		Editing level Site1	Site2	Site3
D. mel	ATC	TAT	CCC	… …	CTG	GTA	ATC	0.85	0.77	1
D. sim	ATC	TAT	CCC	… …	CTG	GTA	ATC	0.88	0.67	0.96
D. sec	ATC	TAT	CCC	… …	CTG	GTA	ATC	0.93	0.63	1
D. ere	ATC	TAT	CCC	… …	CTG	GTA	ATC	0.98	0.59	0.97
D. pse	---	---	---		---	---	---	Exon Deletion		
D. wil	ATC	TAT	CCC	… …	CTG	GTA	ATC	1	0.23	1
D. moj	ATC	TAT	CCC	… …	CTG	GTA	ATC	0.92	0.45	0.96
B. ter	ATC	TAC	CCC	… …	CTG	GTA	ATC	0.93	0.97	0.98
A. mel	ATC	TAC	CCC	… …	CTG	GTA	ATC	0.31	0.79	0.81
A. ech	ATC	TAC	CCC	… …	CTG	GTA	ATC	0	0	0
	site1				site2, 3					

B

***qvr* (*quiver*)**

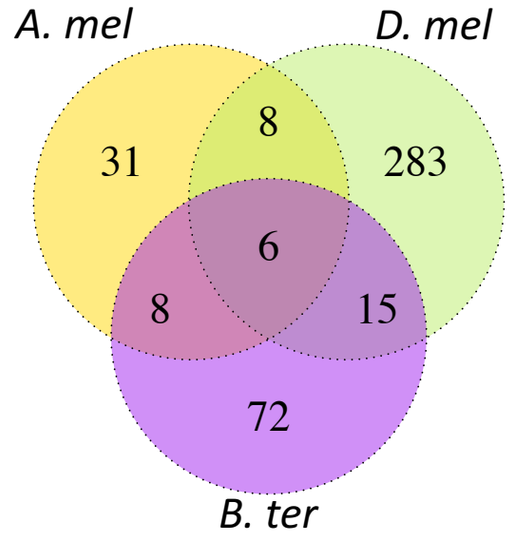
	Ser>Gly					His>Arg			Editing level Site1	Site2
D. mel	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.88	0.61
D. sim	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.96	0.60
D. sec	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	1	0.78
D. ere	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.96	0.82
D. pse	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.99	0.84
D. wil	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.98	0.80
D. moj	GAA	AGT	TCA	GGC	AAT	GGA	CAT	ATG	0.96	0.86
B. ter	GAA	AGT	ACC	GGC	ACT	GGT	CAC	ATG	0.88	0.79
A. mel	GAA	AGT	ACC	GGC	ACT	GGT	CAC	ATG	0.50	0.34
A. ech	GAA	AGT	ACC	GGC	ACT	GGT	CAC	ATG	0.87	0.67
	site1					site2				


A
Editing level
Workers
Drones
0.0
0.2
0.4
0.6
0.8
1.0
B
P = 0.047
Adar expression
0
200
400
600
800
Nurses
Foragers
C
Rho = 0.57
P = 0.049
Cumulative editing level
50
55
60
65
70
400
500
600
700
800
Adar expression
Nurses
Foragers
D
Editing level
Foragers
Nurses
E
Editing level
PC2
PC1
−2.0
−1.0
0.0
1.0
−1
0
1
2
3
Drone
Nurses
Foragers
F
Gene expression
PC2
PC1
−50000
−20000
0
−5000
10000
25000
Drone
Nurses
Foragers

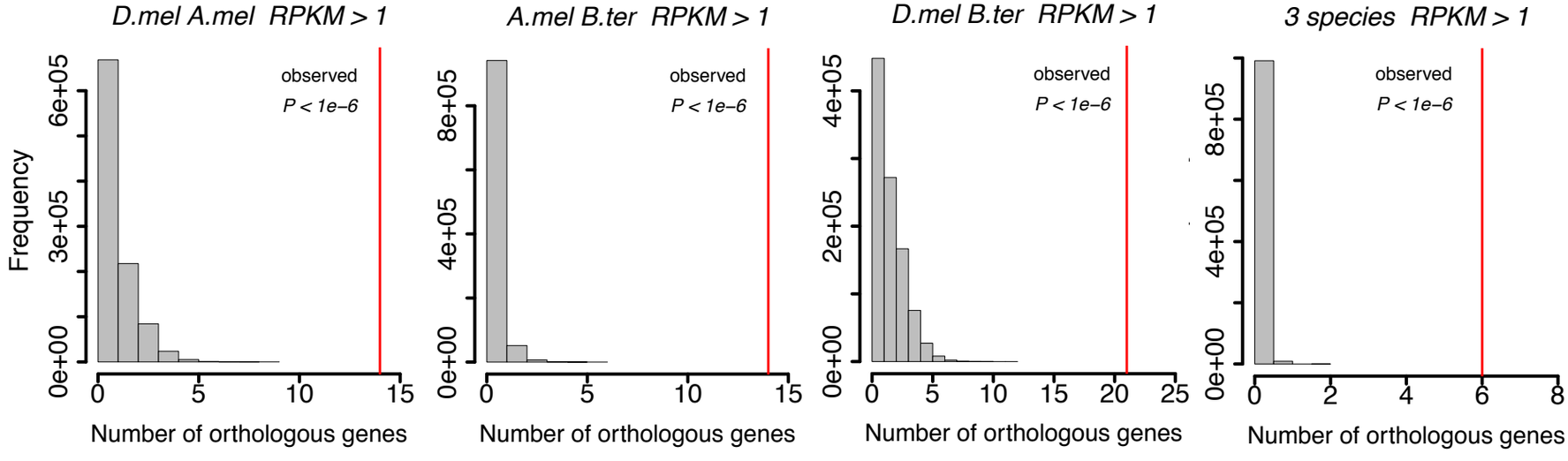
A

Adar	site1 Ser>Gly	site2 Ile	Editing level site1	Editing level site2
D. mel	TTT **A**GT CCT	AAA ATT GCC	0.33	\
D. sim	TTT **A**GT CCT	AAA ATT GCC	0.22	\
D. sec	TTT **A**GT CCT	AAA ATT GCC	0.47	\
D. ere	TTT **A**GT CCT	AAA ATT GCC	0.43	\
B. ter	TTT TCA CCT	AAA AT**A** GCT	\	0.45
A. mel	TTT TCA CCA	AAA AT**A** GCT	\	0.42
	Ser	Ile>Met		

B



C



D

	N	*S*	Observed *N*/*S*	Expected *N*/*S*	*P* value
Sites in genes shared by at least two species					
D. melanogaster	127	22	5.77	3.80	0.038
A. mellifera	37	3	12.33	2.26	4.5E-4
B. terrestris	76	21	3.62	2.29	0.036
Sites in species-specific edited genes					
D. melanogaster	539	117	4.61	3.80	0.031
A. mellifera	74	6	12.33	2.26	5.2E-7
B. terrestris	87	35	2.49	2.29	0.38

Nonsynonymous editing sites in honeybees were under positive selection

Differential editing may contribute to the phenotypic diversity between sub-castes

Target genes acquire editing in different clades, suggesting convergent evolution