Title:

The evolutionary dynamics of the odorant receptor gene family in corbiculate bees

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Abstract

Insects rely on chemical information to locate food, choose mates, and detect potential predators. It has been hypothesized that adaptive changes of the olfactory system facilitated the specialization and diversification of numerous insect lineages. In fact, modifications of life history strategies often occur in parallel with evolutionary changes in Odorant Receptor (OR) genes. However, the molecular mechanisms underlying the evolution of insect OR genes remain poorly understood. Corbiculate bees include economically and ecologically important bee pollinators that exhibit varying degrees of social organization, diverse sex pheromone systems, and manifold associations with floral resources. We investigated the molecular mechanisms governing the evolution of the OR gene family in corbiculate bees. Our results indicate that the genomic organization of the OR gene family has remained highly conserved for approximately 80 million years, despite exhibiting major changes in repertoire size among bee lineages. Moreover, the evolution of OR genes appears to be driven by lineage-specific gene duplications in few genomic regions harboring large numbers of OR genes. A selection analysis revealed that OR genes evolve under positive selection, with the strongest signals detected in recently duplicated copies. Our results indicate that chromosomal translocations had a minimal impact on OR evolution, and instead show that local molecular mechanisms drive OR repertoire size. Our results provide empirical support to the longstanding hypothesis that positive selection shaped the diversification of the OR gene family. Our results shed new light on the molecular mechanisms underlying the evolution of olfaction in insects and gene families in general.

Significance Statement

Despite playing a central role in the diversification of insect lineages, the evolution of insect olfactory systems remains poorly understood. Here, we analyze the Odorant Receptor (OR) repertoire across the corbiculate bees—a lineage of important insect pollinators—to test the hypothesis that genomic re-organization and positive selection shaped the evolution of the OR gene family. We show that locally acting molecular mechanisms are the driving force of OR gene family evolution in corbiculate bees. Moreover, we provide empirical support for the longstanding hypothesis that positive selection impacts the evolution of recently duplicated insect OR genes. Our results augment our understanding of the molecular mechanisms that govern the evolution of olfactory systems in insects and gene families in general.

Introduction

Animals have evolved sophisticated sensory systems that can detect and discriminate airborne volatile chemicals and provide precise information about food, enemies, and mating partners (1). Insects detect olfactory signals and cues via olfactory sensory neurons located in the antenna. Each olfactory sensory neuron expresses Odorant Receptor (OR) proteins on the cell membrane (2-4). It is the interaction of volatile odorant molecules with these OR proteins that initiates the signal transduction and transmission toward the olfactory centers in the insect brain (2, 4). Insect genomes are endowed with a diverse array of functional OR genes, each of which encodes a unique odorant receptor protein tuned to a specific set of odors (5-7). The entire repertoire of OR genes expressed in the olfactory organs determines the spectrum of chemical volatiles that an insect species can detect.

Comparative analyses of insect genomes have revealed that the number of OR genes and divergence among them can vary widely between species. Insect genomes may contain as few as 10 OR genes (head lice, (8)) and as many as 300 OR genes (ants, (9, 10)). In fact, the expansion and contraction of the OR gene family has been linked to shifts in the sensory ability of some insect lineages. This supports the idea that adaptation to novel food resources (11-13) or modifications in the pheromone communication system (14) can be mediated by peripheral changes in the OR gene repertoire. In addition, changes in the amino acid sequence of existing OR genes have been shown to correspond to adaptive shifts in sensory tuning (15, 16). However, we lack a clear understanding of the specific genetic and molecular mechanisms that drive the diversification of the insect OR gene family (17, 18).

Gene family evolution is determined by multiple molecular mechanisms including genomic drift, natural selection, and chromosomal rearrangements. Birth-death processes can directly impact the number of genes within a gene family (19). For example, genomic drift may generate new gene copies through gene duplication (gene 'birth') while existing copies can be purged via pseudogenization or deletion (gene 'death'). Novel mutations resulting in gene duplication or gene loss may be subsequently fixed through neutral genetic drift or positive selection (17, 19, 20). Although OR gene repertoire sizes have been analyzed extensively in various insect lineages, the relative contribution of positive selection and neutral processes in a birth-death evolutionary framework remains uncertain (17, 18).

The genomic organization and architecture of gene families may contain signatures of past evolutionary forces that shaped their diversification. For instance birth-death processes often produce tandem arrays of OR genes, which are groups of ancestrally duplicated genes located in close physical proximity along the genome (19, 21). Chromosomal rearrangements and transposition, on the other hand, can lead to inter-chromosomal translocations of OR genes (22, 23), and it has been hypothesized that changes in the location of OR genes along the genome can influence their evolution (18, 23, 24). Although both tandem duplications and

139 chromosomal rearrangements may influence the evolution of the OR gene family,
140 the relative contribution of each process remains unknown.
141
142 Like most insects, bees rely on olfactory information to regulate a wide array of
143 behaviors, including the location of food sources and nesting materials, the
144 identification of mating partners, and social interactions with other colony members
145 in social species. Corbiculate bees encompass a group of ecologically and
146 economically important bee lineages, including honey bees, bumble bees, stingless
147 bees, and orchid bees. Honey bees are estimated to pollinate one-third of
148 agricultural crops (25), and stingless bees and orchid bees are the major pollinators
149 of numerous tropical flowering plant species (26, 27). Corbiculate bees have evolved
150 a variety of phenotypic and behavioral traits that require specialized olfactory
151 functions. These include the evolution of an obligate cooperative social lifestyle
152 (eusociality), which has resulted in the evolution of specialized pheromone
153 communication systems in honey bees, bumble bees and stingless bees (28-31). In
154 addition, orchid bees evolved a unique pheromone communication system in which
155 male bees concoct species-specific perfume bouquets from scents collected from
156 flowers and other sources to subsequently use during courtship display (32-34).
157 However, despite the central role that olfactory encoding plays in the biology of
158 corbiculate bees, we lack a clear understanding of the evolution of the OR gene
159 family in this diverse group of bees.
160
161 Here we analyzed the evolution of the OR gene family in a set of species including
162 the four corbiculate bee clades, spanning 80 million years (my) of evolution. We
163 tested whether the evolution of the OR gene family has been influenced by changes
164 in the genomic organization of genes, as hypothesized based on patterns observed
165 in *Drosophila* and mammalian genomes (18, 23, 24). Second, we tested the
166 hypothesis that birth-death processes, in combination with positive selective
167 pressures, promoted the origin of novel OR genes. Finally, we estimated the relative
168 importance of positive selection in shaping OR sequence divergence, a mechanism
169 that has been hypothesized to drive sensory adaptation in insect lineages (15, 35,
170 36).
171
172 **Results**
173
174 ***The Evolution of Corbiculate Bee Odorant Receptors is Highly Dynamic.*** To
175 analyze the evolutionary dynamics of odorant receptor (OR) gene repertoires in
176 corbiculate bees, we reconstructed the phylogenetic relationships among the entire
177 OR gene family based on both whole-genome sequences and transcriptome data
178 obtained from ten bee species (Fig. 1). An initial comparison of OR annotation based
179 on genomic and transcriptomic data indicated no differences in annotation
180 accuracy, but as expected, the number of genes tended to be lower for
181 transcriptome data (Supplemental Text). Our analysis included published whole-
182 genome sequence data from at least one species per corbiculate bee tribe (21, 37-
183 39). In addition, we sequenced the genome of one orchid bee (*Euglossa dilemma;*
184 (40)) and the antennal transcriptomes of five additional orchid bee species. The

resulting dataset, which included three orchid bee species pairs, allowed us to analyze the evolution of the OR gene family at multiple taxonomic levels over a broad range of divergence times (0.15 to 80mya; Fig. 1). We compared the evolution of the OR gene family, the largest and most dynamic chemosensory gene family in insects, to the evolution of four additional chemosensory gene families, including the Gustatory Receptors (GRs), Ionotropic Receptors (IRs), Odorant Binding Proteins (OBPs), and Chemosensory Proteins (CSPs). We used genomic data only to derive evolutionary patterns of gene family size since transcriptome data often yield incomplete gene sets (Fig. 1).

Gene repertoire size varied substantially among bee lineages, ranging from 142 OR genes in the orchid bee *Eufriesea mexicana* to 196 OR genes in the stingless bee *Melipona quadrifasciata* (Fig. 1). The identified ORs were organized in 115 homologous OR ortho-groups consisting of orthologs and/or inparalogs (*i.e.* lineage-specific expansions, Fig. 2, Fig. S1). Of these ortho-groups, 24 did not contain duplications but instead consisted of simple 1:1 orthologous genes that were present in three or more bee species (Fig. 2b). The remaining 91 ortho-groups included species-specific, genus-specific, and tribe-specific duplications or larger expansions (Fig. 2c). In contrast to the other four gene families analyzed, the repertoire size of ORs is highly variable among corbiculate bee lineages. The majority of genes in the non-OR gene families revealed simple 1:1 orthology with few lineage-specific duplications resulting in similar repertoire sizes among bee lineages (Fig. 1; Fig. S2). An exception to this was an observed increase in gene family size in honey bee OBPs as well as bumble bee GRs (Supplemental Text). Among all chemosensory genes, we identified 32 gene duplications and large expansions in the honey bees, 36 in the bumble bees, 26 in the stingless bees, and 18 in the orchid bees, most of which were observed in the OR gene family (89%, Tab. S1). These results show that the evolution of the corbiculate bee OR gene family repertoire is characterized by highly dynamic lineage-specific gene expansions.

The Genomic Organization of the OR Gene Family is Highly Conserved in Corbiculate Bees. Next, we investigated the genomic organization of OR genes across the corbiculate bees. Our analysis revealed that OR genes are widely distributed throughout the five bee genomes we analyzed. This supports previous studies where OR genes were detected on scaffolds assigned to almost all chromosomes in those species with available linkage groups (*i.e.* honey bees and bumble bees; (21, 37, 38)). Congruent with a birth-death process for OR gene evolution, the majority of genes were clustered in large tandem arrays and only few ORs were found in isolated genomic regions as singletons. We tested whether the chromosomal location of OR genes is conserved among corbiculate bees by comparing the 200 kb flanking regions of all OR singletons and tandem arrays among species using an “all-against-all” reciprocal blastn approach.

With the exception of a small number of ancestral translocations (Supplementary Text), we found that the flanking regions and thus the physical chromosomal locations of most OR genes were strictly homologous in all corbiculate bee species

231 (Tab. S2). Furthermore, all orthologous OR genes found in two or more species were
232 located in homologous chromosomal regions. Paralogous genes within species-
233 specific, genus-specific, and tribe-specific duplications and larger OR sublineage
234 expansions were always located in the same tandem array. Similarly, ORs located
235 within the same genomic region were generally more closely related to each other
236 than ORs located in different genomic regions that formed highly supported
237 monophyletic gene clades (Fig. 2a). On the basis of this strong correlation between
238 phylogenetic relationship and genomic location, we defined a total of 25 well-
239 supported subfamilies in the OR gene family that largely corresponded to groups
240 previously identified in ants (Fig. 2a; (41, 42)). In addition to the high conservation
241 of genomic locations of OR genes and tandem arrays, we found that the organization
242 of genes within tandem arrays (*i.e.* gene synteny) is also highly conserved
243 (Supplementary Text; Fig. S3). Our analysis identified similar patterns of
244 conservation in the genomic organization of other chemosensory gene families,
245 including Gustatory Receptors, Ionotropic Receptors, Odorant Binding Proteins, and
246 Chemosensory Genes (Dataset S1).
247
248 ***The Evolution of OR Repertoire Sizes is Governed by Dynamic Evolutionary***
249 ***Changes in Large Subfamilies.*** Our analysis revealed that although the genomic
250 organization of OR tandem arrays is highly conserved across corbiculate bee
251 lineages, OR evolution is highly dynamic, with multiple taxon-specific OR subfamily
252 expansions. In order to investigate potential localized molecular mechanisms that
253 shape the evolution of the OR gene family, we analyzed the divergence of the 25 OR
254 subfamilies (see above). We found that variation in subfamily size among species
255 was positively correlated with mean subfamily size (Fig. 3a, Pearson coefficient
256 r=0.85, p=0; Orchid bees only: r=0.6, p=0.0013), a common pattern in gene family
257 evolution (Dennis et al. 2017). This result suggests that larger OR subfamilies have a
258 higher gene turnover than smaller gene subfamilies. Accordingly, all subfamilies
259 with one or two genes per species consisted of simple 1:1 orthologous genes across
260 the corbiculate bee genomes and transcriptomes (Fig. S1; Tab. S3).
261
262 Three of the OR subfamilies that exhibited high interspecific variation in OR
263 repertoire size had an expansion in a single bee species. The G04A subfamily was
264 expanded in the honey bee *A. mellifera* (Fig. 2c), the G09A subfamily was expanded
265 in the orchid bee *Ef. mexicana,* and the G09C subfamily was expanded in the
266 stingless bee *M. quadrifasciata* (Fig. 3b, Tab. S3). Several subfamily expansions were
267 observed in two or more species. Although most expansions are not shared by
268 multiple bee species with similar life history traits, the G02A subfamily was
269 expanded in all obligate eusocial bee species we included in our analysis (honey bee,
270 bumble bee and stingless bee; Fig. 3b). Conversely, ORs in the subfamily GUnA and
271 G12A were expanded in orchid bees (Fig. 3b, Tab. S3), which are known for their
272 unique perfume collecting behavior. The expansion we observed in the subfamilies
273 G02A and GUnA correspond to well-supported lineage-specific clusters of paralogs
274 in our phylogenetic analysis (Fig. S1), suggesting that these subfamilies are
275 independent expansions leading to elevated OR numbers. In contrast, more than
276 half of the orchid bee OR expansion in subfamily G12A was shared between the

277 orchid bee species included in our analysis (*i.e.* orthologous copies were present),
278 suggesting that the expansion was already present in their common ancestor.
279
280 ***Positive Selective Pressures Drive the Evolution of Both Paralogous and***
281 ***Orthologous OR Genes.*** To investigate the selective pressures that shaped the
282 evolution of the corbiculate bee OR gene family, we directly compared the evolution
283 of ORs with the evolutionary patterns of the remaining major chemosensory gene
284 families, including the GRs, IRs, OBPs, and CSPs. We classified all annotated
285 chemosensory genes into orthologous groups consisting of at least four orthologs
286 and/or inparalogs based on our phylogenetic analysis (see Fig. 2b-c for examples of
287 orthologous groups). The resulting 159 orthologous groups (115 OR, 13 GR, 9 IR, 15
288 OBP, and 7 CSP orthologous groups) contained a total of 1588 chemosensory genes
289 (Dataset S1). We tested each of the orthologous groups for the presence of episodic
290 positive selection using branch-site model based dN/dS estimates (Smith et al.
291 2015). We identified 54 chemosensory genes (2.1% of all terminal branches) and 57
292 deeper branches (6.1% of 941 deep branches) with signatures of positive selection
293 under a 5% false discovery rate (FDR). This corresponds to 4.4% of all branches
294 tested. We found no correlation between divergence time and positive selective
295 pressures (Supplemental Text; Tab. S4). Furthermore, there was no difference in the
296 number of branches under positive selection between the four corbiculate bee
297 tribes (Tab. 1). However, a comparison across gene families revealed a higher
298 proportion of branches under positive selection in the GRs relative to any other
299 gene family in all bee lineages, supporting the hypothesis that GRs exhibit elevated
300 signatures of positive selection across many insect groups ($p<0.0001$; Fisher's Exact
301 Test; Supplementary Text) (12).
302
303 Those branches that exhibited signatures of positive selection were distributed
304 throughout the entire OR gene tree (Fig. S1). Interestingly, only 32% of all branches
305 that we identified to evolve under positive selection belonged to lineage-specific
306 duplications or larger gene expansion events (Tab. S5). Thus, the majority of
307 branches under positive selection correspond to orthologous rather than
308 paralogous gene copies. This pattern held true when we considered only those five
309 species with genomic data. However, when normalized by the total number of the
310 two branch classes (duplication and non-duplication branches, respectively), we
311 found that a higher proportion of all tested duplication branches were under
312 positive selection in comparison to non-duplicated branches (6% vs. 3.9%, Fisher's
313 Exact Test, $p< 0.05$, Tab. S5). Together, these results suggest that positive selective
314 pressures play an important role in the evolution and divergence of duplicated OR
315 genes.
316
317 In addition, we observed that 10 of the 11 OR subfamilies without any branches
318 under positive selection included simple (single-copy) 1:1 orthologs only
319 (Subfamilies G01A, G01B, G02B, G05A, G09D, G14B, G15A, G15B, GUnB, ORco; Fig.
320 S1). This observation suggests the existence of a core set of OR genes with
321 conserved chemosensory functions across all corbiculate bees.
322

Discussion

Our analysis focused on documenting signatures of positive selection as well as changes in the genomic organization of genes in the OR gene family analyzing 1394 ORs spanning multiple evolutionary time scales across the corbiculate bees. Our analysis revealed that the corbiculate bee OR gene family is highly dynamic (Fig. 1, Fig. 2, Fig. S1), a pattern consistent with that observed in other insect lineages. However, the genomic organization we identified in corbiculate bees differs from that previously reported in other insect groups. First, we found that the genomic organization of OR genes is highly conserved between corbiculate bee lineages, despite sharing a common ancestor 80 million years ago (Tab. S2). Second, OR gene repertoire sizes vary significantly between bee species due to lineage-specific expansions in a few large tandem arrays, some of which correlate with the evolution of obligate eusociality and pheromone communication systems (Fig. 3; Fig. S2). These findings suggest that the highly dynamic evolutionary history of the OR gene family is independent of changes in genomic organization and instead more likely influenced by locally acting molecular mechanisms. Furthermore, we found evidence for elevated rates of positive selection on duplicate ORs (Tab. 1; Tab S6), supporting the main prediction from the birth-death model of gene family evolution. Finally, we identified a fraction of bee ORs under positive selection that have not undergone previous duplication, highlighting the importance of positive selective pressures in OR sequence divergence, possibly in response to ecological adaptation.

Locally Acting Molecular Mechanisms are the Driving Force of Chemosensory Evolution. Our phylogenetic and genome annotation analysis revealed a high degree of genomic landscape conservation in the OR gene family across the corbiculate bees. This pattern was accompanied by a fast diversification rate of OR genes, indicating that genomic rearrangements had a relatively weak influence relatively to the stronger influence of local molecular mechanisms. The high degree of conservation in the overall genomic organization holds true even between corbiculate bee lineages that shared a common ancestor 80 million years ago. This is an unprecedented level of genomic conservatism in OR organization, and is particularly surprising given the diverse factors known to affect genome composition and genome architecture. In fact, the bee lineages we examined exhibit more than two-fold variation in chromosome number (9 in stingless bees to 20 in orchid bees; (43)), an order of magnitude of variation in genome size (ranging from 0.25GB in honey bees to 3GB in orchid bees; (37-40)), as well as exceptionally high recombination rates (44-46). Moreover, the high level of conservation stands in sharp contrast to known evolutionary mechanisms that affect genomic organization of OR genes in drosophilid flies, the only other insect group for which the evolution of chemosensory genes has been examined in comparable detail. The genomes of drosophilid flies exhibit complex genome rearrangements leading to a high degree of ortholog OR gene translocation within evolutionary time scales similar to those spanning the bee species we analyzed (22, 47, 48). A similarly dynamic pattern of OR tandem array architecture was previously observed in mammalian genomes (49). These observations lead to the hypothesis that genomic rearrangements are

important in the evolution of chemosensory receptors across vertebrate and invertebrate lineages (18, 23, 24). In contrast, our results show that the evolution of the OR gene family in corbiculate bees has resulted in highly divergent gene repertoires without undergoing genome rearrangements.

Our results revealed no correlation between the evolution of genomic organization and genome size. In fact, genome size of most corbiculate bees resembles those reported for drosophilid flies (37, 50). Instead, a recent comparison of the genomes of honey bees and bumble bees revealed high conservation of genome synteny between these species (44), indicating that conserved genomic architecture of OR tandem arrays may result from a low precedence of genome rearrangements in corbiculate bees. Indeed, our analysis revealed that synteny among OR genes in orchid bees is almost perfectly conserved even within highly dynamic genomic areas that are dominated by gene duplication (Supplementary Text). Despite the high level of conservation in the genomic organization reported here, we find a high degree of OR gene divergence and turnover among corbiculate bees. This result suggests that chemosensory gene evolution in corbiculate bees, and perhaps insects in general, is shaped by local rather than global mechanisms of genome evolution.

Large Tandem Arrays are Hotspots for the Evolution of Novel ORs. The high rate of variation we observed in OR repertoire size among lineages, along with the prevalence of lineage-specific gene expansions in large OR tandem arrays, suggests that large gene subfamilies play an important role in the evolution of novel OR genes in corbiculate bees. Insect OR repertoires are highly dynamic between lineages in comparison to other chemosensory gene families (18, 51). Our results confirm this pattern for corbiculate bees. In contrast to previous studies, however, we find that these expansions are driven by lineage-specific expansions within large tandem arrays harboring entire OR subfamilies. This result is congruent with a recent phylogenetic analysis that showed that gene duplication and pseudogenization events of OR genes in Hymenoptera are often found in large subfamilies (52). The increased gene turnover rate could be influenced by elevated rates of genome replication errors resulting from non-allelic homology of a large number of closely related paralogous loci in close proximity within large tandem arrays (53-55). Nevertheless, other factors such as transposable element activity and the timing of DNA replication can influence segmental duplication rates (53, 56). Independent of the underlying genomic mechanism, our results suggest that large OR tandem arrays are a major source of novelty in chemosensory receptors in corbiculate bees and potentially other Hymenoptera. Accordingly, large tandem arrays are likely an important source of functional novelty associated with the detection of novel molecules or blends of chemical compounds. Hence, it is possible that the gene expansions we identified coincide with key evolutionary innovations such as complex social chemical communication in the obligate eusocial honey bees, bumble bees and stingless bees or perfume-collecting behavior in orchid bees.

It has been hypothesized that OR gene family size is correlated with the degree of complexity in the chemical communication system displayed by eusocial

415 Hymenoptera (57). Although our data do not support this general trend, we find a
416 positive correlation between OR gene subfamily size and the presence of obligate
417 eusociality. For instance, the OR subfamily G02A is expanded in all three obligate
418 eusocial bee species analyzed, relatively to the smaller size exhibited by the solitary
419 to weakly social orchid bees. Moreover, the OR subfamily G02A is expanded in ants
420 but not in the facultative social halictid bee *Lasioglossum albipes*, which exhibits
421 subfamily G02A gene numbers comparable to orchid bees (Subfamily L in (42, 52)).
422 Thus, it is possible that ORs in subfamily G02A play a central role in the olfactory
423 detection and processing of pheromones involved in social behavior. Similarly, an
424 expansion of the '9-exon' subfamily (here subfamilies G04A, G05B, G11A, G11B, and
425 G12A; Fig. 2a; Fig. 3b; Fig S1) was hypothesized to be important in the evolution of
426 eusociality due to a putative role of '9-exon' ORs in the detection of social
427 pheromones (namely cuticular hydrocarbons, (9, 10, 58)). We find an expansion of
428 the '9-exon' subfamily in the eusocial honey bees and bumble bees but not eusocial
429 stingless bees. This observation contrasts with the previous suggestion that the
430 expansion of this OR subfamily was concomitant with the evolution of eusociality in
431 Hymenoptera. However, it is possible that ORs involved in social pheromone
432 communication evolved independently in different Hymenoptera lineages. Future
433 studies should investigate the possibility that olfactory adaptations to social
434 behavior evolved through multiple analogous routes in social Hymenoptera (39,
435 59).
436
437 In addition, we found two OR subfamilies that exhibit an expansion in the orchid bee
438 lineage (namely GUnA and G12A). While large expansions were in general species-
439 specific clusters of paralogous ORs (*e.g.* Fig. 2c; Fig. S1), more than half of the ORs in
440 the orchid bee expansion of subfamily G12A were orthologous ORs found in all the
441 seven species included in our analysis. This shared expansion may correspond to an
442 orchid bee specific olfactory specialization. Since the only known olfactory behavior
443 clearly distinguishing orchid bees from all other corbiculate bees is male perfume-
444 collecting behavior (34, 60), it is possible that the G12A subfamily is involved in the
445 detection and encoding of these exogenous compounds. Functional analyses are
446 required to investigate the olfactory coding mediated by members of this OR
447 subfamily.
448
449 ***Selective Patterns of Corbiculate Bee Chemosensory Genes Support the Birth-***
450 ***Death Model of Gene Family Evolution.*** It has been proposed that chemosensory
451 gene families evolve under a birth-death process where duplications of existing
452 genes give rise to new gene copies (gene 'birth') whereas pseudogenization purges
453 genes from the genome (gene 'death'; (19)). The birth-death model predicts a
454 relaxation of purifying selective pressures after duplication allowing subsequent
455 diversification of one or both copies through positive selection for newly
456 accumulated beneficial mutations. Accordingly, the evolution of highly dynamic
457 chemosensory gene families is likely driven by the action of positive selection.
458 Nevertheless, while previous studies detected a high impact of gene duplication and
459 loss on chemosensory gene family evolution, there is very little evidence for

460 diversification through positive selection (12, 22, 52, 61-63), even in large lineage-
461 specific gene family expansions (42).
462
463 Our analysis provides empirical evidence congruent with the selective pressures
464 predicted by the birth-death model of chemosensory gene family evolution. We
465 show that positive selective pressures are stronger on duplicated chemosensory
466 genes than in orthologous chemosensory genes across corbiculate bees. The
467 observed higher proportion of branches under positive selection is in contrast to
468 previous analyses of insect chemosensory genes and might be partly explained by
469 the former use of less powerful algorithms, reliance on a priori expectations,
470 incomplete testing, or differences in taxon sampling (64). It is thus unclear whether
471 the disparity between studies reflects technical differences or differences in
472 chemosensory gene evolution between different groups of insects. Regardless, our
473 findings suggest a non-negligible impact of positive selective pressures on
474 chemosensory gene evolution in corbiculate bees.
475
476 ***Diversification of Orthologous ORs through Positive Selection Drives Divergence***
477 ***in Chemosensory Systems.*** Our selection analysis indicates that positive selection is
478 involved in the divergence of orthologous ORs, providing an evolutionary
479 mechanism for the diversification of chemosensory genes independent of gene
480 duplication. We found 54 ortholog chemosensory genes and deeper branches that
481 exhibit signatures of positive selection. Moreover, our analysis using three pairs of
482 sympatric species revealed the presence of orthologous chemosensory genes
483 evolving under positive selection irrespective of divergence times (0.15 - 13 my).
484 This result suggests that positive selection is an important evolutionary force
485 driving the diversification of orthologous chemosensory genes in corbiculate bees.
486 Similarly, Leary et al. (2012) (15) showed that positive selection of specific amino
487 acid substitutions in OR orthologs led to novel receptor functions through changes
488 in ligand binding affinities in a lineage of moths. Under this model, orthologous OR
489 genes undergo functional divergence driven by positive selection in the absence of a
490 duplication event. While OR gene duplications are more common in large
491 subfamilies in corbiculate bees, this mechanism might be important for the
492 evolution of interspecific functional divergence in smaller subfamilies. Thus, our
493 results suggest that positive selection is not only important in driving divergence
494 between OR duplicates within taxa, but also orthologous ORs between taxa. It is also
495 possible that a combination of these two mechanisms drives the evolution of
496 chemosensory genes in other insect lineages.
497
498 ***Evidence for ORs with Core Chemosensory Function in Corbiculate Bees.*** We
499 detected the existence of multiple highly conserved OR lineages that appear to be
500 subject to strong purifying selection. This observation supports the existence of a
501 core set of OR genes with conserved functions across all corbiculate bees and
502 perhaps other more distantly related bee lineages. We detected 10 OR subfamilies
503 and 14 orthologous groups within larger subfamilies that solely consisted of 1:1
504 orthologs. While most OR subfamilies and orthologous groups were characterized
505 by taxon-specific duplications and divergence driven by positive selective pressures,

our results suggest that these OR lineages are highly conserved throughout at least 80 million years of evolution. This observation implies that corbiculate bee genomes contain a core set of olfactory genes with conserved chemosensory functions. In fact, high conservation of OR genes is typical for some species with highly similar natural history (12, 47, 65). Detailed functional analysis of these core OR genes may elucidate the role of conserved olfactory functions in corbiculate bees of ecological importance.

Conclusion

Odorant receptors (ORs) have been hypothesized to play an important role in chemosensory adaptation, for example by enabling the evolution of novel pheromone detection systems associated with social communication or mate attraction (1, 66). However, little is known about the molecular mechanisms driving the evolution of insect OR gene families. Our analysis of the OR gene family in an important lineage of insect pollinators, the corbiculate bees, elucidated the role of positive selective pressures and genomic organization on the evolution of the OR gene family.

We show that OR gene family size is highly dynamic between corbiculate bee species. In contrast, the underlying genomic organization of OR genes is highly conserved among lineages over at least 80 million years. Consistent with these findings, ORs are arranged in tandem arrays located in close proximity along the genome. Moreover, the genomic location correlates with the phylogenetic relationships among genes that fall in 25 well-supported OR subfamilies. We find that a few large subfamilies contain a considerable amount of lineage-specific gene duplications, explaining most of the variation in gene family size. Further, we show that ORs evolve under strong positive selective pressures, with a higher impact on duplicated genes. Our study suggests that locally acting molecular mechanisms are the driving force of OR gene family evolution in corbiculate bees. Moreover, we provide empirical support for the longstanding hypothesis that positive selection is an important mechanism in the evolution of duplicated OR genes in insects. These results elucidate the OR gene family evolution in corbiculate bees and extend the current understanding of OR gene family evolution to other insect groups.

Methods

Sampling and Sequencing

Sampling and sequencing procedures followed Brand et al. 2015. Briefly, males of the four orchid bee species *Euglossa flammea*, *Eg. imperialis*, *Eulaema bombiformis* and *El. meriana* were sampled at the La Gamba Field Station in Costa Rica using different chemical baits (67). Antennal transcriptomes for each species were based on pools of the antennae of 20 individuals for the *Euglossa* species and 5 individuals for the *Eulaema* species. Bees were chilled on ice and the antennae of each torpid male were dissected using sterile forceps and transported either on liquid nitrogen

551 or RNA-later (Thermo Fisher Scientific, Waltham, MA) and kept frozen until RNA-
552 extraction.
553
554 Total-RNA was extracted and quantified as described (35). Barcoded cDNA libraries
555 were prepared using the NEBnext Ultra RNA Library Prep kit for Illumina (New
556 England Biolabs, Ipswich, MA) following the manufacturers protocol and sequenced
557 in two pools of two species on one HiSeq 2500 (*Euglossa*) and one HiSeq 4000
558 (*Eulaema*) lane for 2x100bp cycles. (Raw sequence reads are available at the NCBI
559 Sequence Read Archive XXX).
560
561 **De novo *transcriptome assembly and transcript recovery.***
562 Identical raw reads were merged using fastuniq v1.1 (68) and subsequently
563 trimmed by trim_galore v0.3.7 (Babraham Bioinformatics) if sequencing primers or
564 low-quality bases (Phred-score <20) were detected. The pre-processed reads of
565 each species were used for *de novo* transcriptome assembly following the meta-
566 assembly approach developed in Brand et al. 2015 using the Trinity (69, 70) and
567 SOAPdenovo-trans v2 (71) assemblers. Briefly, each of the two assemblers was run
568 with multiple parameter sets controlling assembly stringency resulting in 25
569 assemblies per species. Trinity settings were set as described (35), whereas
570 SOAPdenovo-trans parameters –e and –d were set to 0, 2, 3, or 5 and 1, 3, 5, or 7,
571 respectively and run in all possible combinations. While Trinity was run with the
572 default single-k-mer mode, SOAPdenovo-trans was run in multi-k-mer mode with
573 nine different k-values (25, 31, 37, 43, 49, 59, 69, 79 and 89).
574
575 ***Annotation of chemosensory genes.***
576 Chemosensory genes of five gene families (ORs, IRs, GRs, OBPs, and CSPs) were
577 annotated in the newly sequenced antennal transcriptomes of the orchid bee
578 species *Eg. flammea*, *Eg. imperialis*, *El. bombiformis*, and *El. meriana*, as well as the
579 publicly available genomes of the orchid bee species *Eg. dilemma* (40) and *Eufriesea*
580 *mexicana* (39), and the stingless bee *Melipona quadrifasciata* (39). Additionally, the
581 CSP gene family was annotated for the bumble bee *Bombus terrestris* (38). All four
582 *de novo* transcriptomes were annotated using a homology-based iterative tBLASTn
583 approach as previously described (35). In order to annotate the genomes, tBLASTn
584 was used to detect scaffolds harboring chemosensory genes with an e-value cutoff of
585 10e-6 based on gene family specific query libraries comprised of published bee,
586 wasp, ant and dipteran OR, IR, GR, CSP and OBP sequences (21, 35, 38, 41, 51, 72,
587 73). Subsequently, the queries were used to predict exon-intron boundaries with
588 exonerate (74) in a scaffold specific manner. All annotations were curated manually
589 and corrected if needed. All annotations can be found in Dataset S1.
590
591 ***Phylogenetic reconstruction of chemosensory gene families.***
592 For each chemosensory gene family we estimated a maximum likelihood (ML) gene-
593 tree using RaxML (75) to infer the genealogical histories of the candidate
594 corbiculate gene family members. On that account, gene family specific protein
595 sequence alignments were constructed using MAFFT (76, 77) applying the L-INS-I
596 algorithm with the --maxiterate option set to 1000 (78). Based on the resulting

597 alignments ML trees were inferred under a JTT + G substitution model for each
598 family with 20 independent ML searches and 100 bootstrap replicates.
599
600 ***Analysis of the genomic organization of chemosensory gene families.***
601 Based on genomic annotations of *Eg. dilemma*, *Ef. mexicana*, *M. quadrifasciata* (this
602 study), *A. mellifera* (21), and *B. terrestris* (38), we analyzed the evolutionary history
603 of genomic structure of chemosensory gene families within the corbiculate bees.
604 Therefore, we applied a reciprocal blast approach that compares the genomic
605 location of all chemosensory gene family tandem arrays and singletons to the
606 genomes of all other species. We required both 200kb flanking regions to be the
607 reciprocal best blast hit (79) in order to call homology of the genomic regions. In
608 case annotations were located less than 200kb away from the end of a scaffold, we
609 used the maximum flanking region length possible, which never corresponded to
610 less than 100kb. The location of ORs in the resulting genomic regions in
611 combination with the gene family phylogeny was used to classify all ORs into
612 subfamilies. Subfamilies were named after the linkage group they were located on in
613 the honey bee genome. Letters were used to allow for the unique indication of
614 subfamilies located on the same linkage group (*e.g.* G12B is the second subfamily
615 found to be located on linkage group 12 of the honey bee). In case a gene family was
616 located on a honey bee scaffold that could not be assigned to a linkage group, it was
617 named 'Un', in short for 'unassigned'.
618
619 In a second approach, we used the OR gene family phylogeny to infer the
620 evolutionary histories of micro-genomic synteny within tandem arrays. Orthologs
621 and paralogs were directly inferred from the ML phylogenies and subsequently
622 mapped to respective genomic coordinates. Since more distantly related species
623 share less orthologous than paralogous gene copies (*e.g.* (21, 52)), we focused on
624 the two orchid bees *Eg. dilemma* and *Ef. mexicana* for this analysis.
625
626 ***Selection analysis***
627 Based on the phylogenetic reconstructions, we defined monophyletic orthologous
628 groups for each gene family consisting of four or more ortholog copies including in-
629 paralogs that were used for all subsequent selection analyses. In order to identify
630 genes under positive selection, we applied the adaptive branch-site relative effects-
631 likelihood (aBSREL) algorithm as implemented in HYPHY (80) to each subgroup to
632 identify branches with dN/dS ratios significantly larger than 1, indicating positive
633 selection along these branches (64). P-values for each aBSREL run were corrected
634 for multiple testing using a false-discovery-rate (FDR) of 5%. In order to analyze
635 patterns of recent gene family divergence in the orchid bees, we conducted pairwise
636 dN/dS analyses for the *Eulaema* species pair and the two *Euglossa* species pairs.
637 Therefore, we extracted orthologous copies from the gene family phylogenies and
638 performed pairwise nucleotide sequence alignments for each orthologous pair.
639 Each alignment was subsequently used for ML estimation of the pairwise dN/dS
640 ratio (model M1) in codeml (81, 82). dN/dS ratios for each pairwise comparison
641 were tested for significant deviations from the neutral expectation (dN/dS=1; model
642 M0) using likelihood-ratio tests.

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858

859 **Figures**

860

861 **Fig. 1** Gene family size dynamics in corbiculate bees. Numbers of previously (*A.*
862 *mellifera, B. terrestris, Eg. viridissima*) and newly annotated (all others) genes for
863 each gene family are indicated. Annotations derived from whole genomes and
864 antennal transcriptomes are labeled. Phylogenetic relationships and divergence
865 times of the ten corbiculate bee species following Ramirez et al. 2010 and Romiguier
866 et al. 2015 are shown. Annotated nodes indicate MRCA of bumble bees and stingless
867 bees (A), honey bees + A (B) and all corbiculate bees (C).

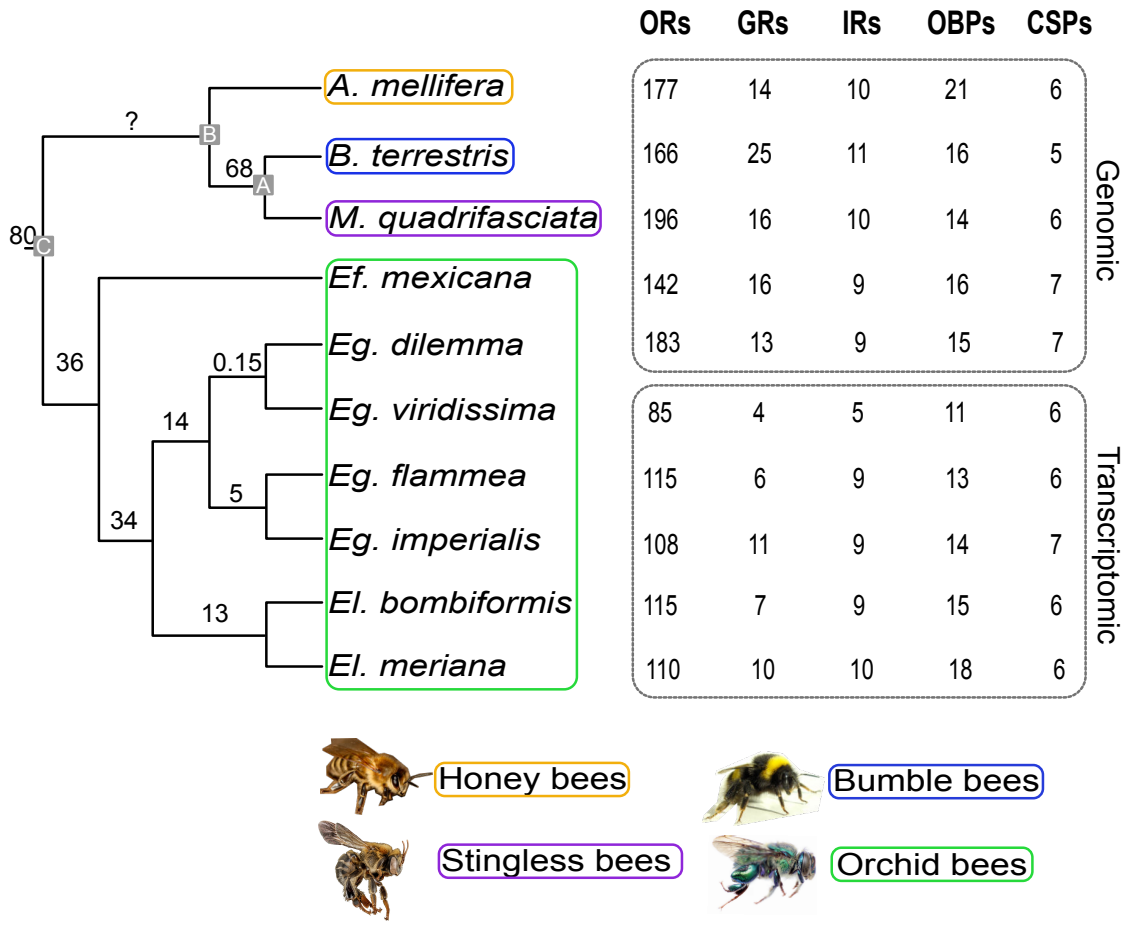
868

869 **Fig. 2** Odorant receptor subfamily dynamics in corbiculate bees. Relationships
870 between subfamilies (**A**) are based on all annotated ORs of 10 corbiculate bee
871 species. Observed differences of within subfamily dynamics are shown with
872 subfamily G13A and subfamily G04A as examples (**B, C**). (**A**) OR subfamilies have
873 high bootstrap support while deeper nodes are of low support. All genes within a
874 subfamily are located in a single tandem array with highly conserved genomic
875 landscapes throughout corbiculate bees. The few exceptions are indicated with *. (**B,**
876 **C**) ORs in subfamily G13A are mainly simple 1:1 single copy orthologs found in all or
877 a subset of the analyzed species (marked '1:1') in contrast to subfamily G04A, where
878 lineage specific duplications and larger expansions in *A. mellifera*, *B. terrestris*, *Eg.*
879 *dilemma* and *Ef. mexicana* dominate (marked 'E'). Branches evolving under positive
880 selective pressures are indicated by a red star. Both, duplicate branches as well as
881 branches in 1:1 ortholog groups were under positive selection. The branch indicated
882 with a * in (**B**) indicates the sistergroup to all other ORs in subfamily G13A which is
883 found at a genomic location different from the rest of the subfamily in all corbiculate
884 bee species representing an ancient translocation event (see text for details).

885

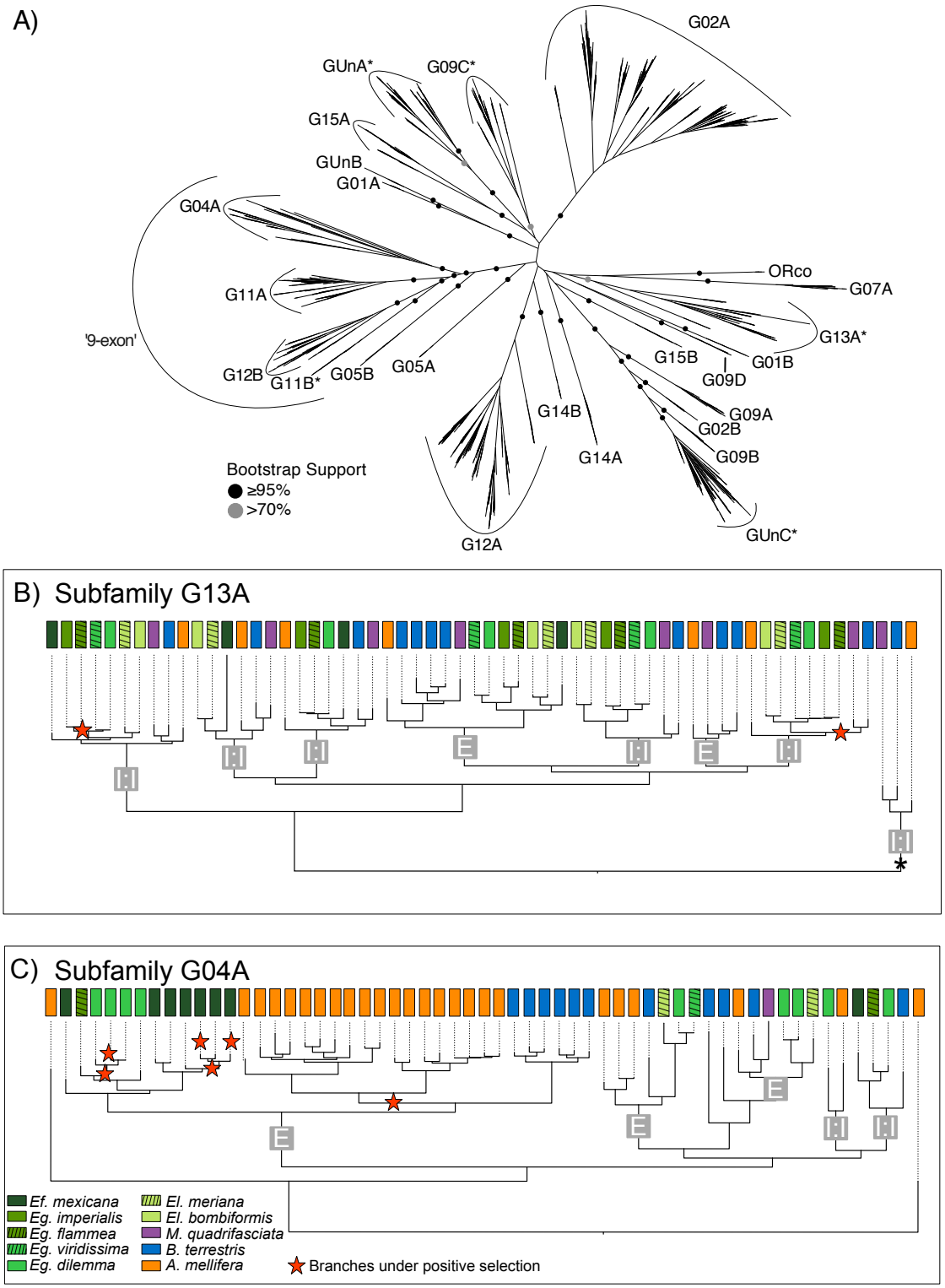
886 **Fig. 3** Evolution of OR subfamily size. (**A**) OR subfamily size variation among
887 corbiculates correlates with mean subfamily size. The pattern holds true for all
888 corbiculates (white dots, black dotted line) and the two orchid bee species alone
889 (black dots, grey dotted line). **(B)** OR subfamily repertoire numbers among
890 corbiculate bees. Numbers of ORs in all 25 subfamilies for the five species with
891 whole-genome data are indicated. Pseudogenes are excluded. *Eg. dilemma* and *Ef.*
892 *mexicana* represent the two orchid bee species. OR subfamily sizes are highly
893 variable between species, while increased subfamily sizes are shared by multiple

894 lineages or occur in single lineages only. 9Exon contains the combined repertoire of
895 subfamilies G04A, G05B, G11A, G11B, and G12A.
896
897
898 **Fig. 1**

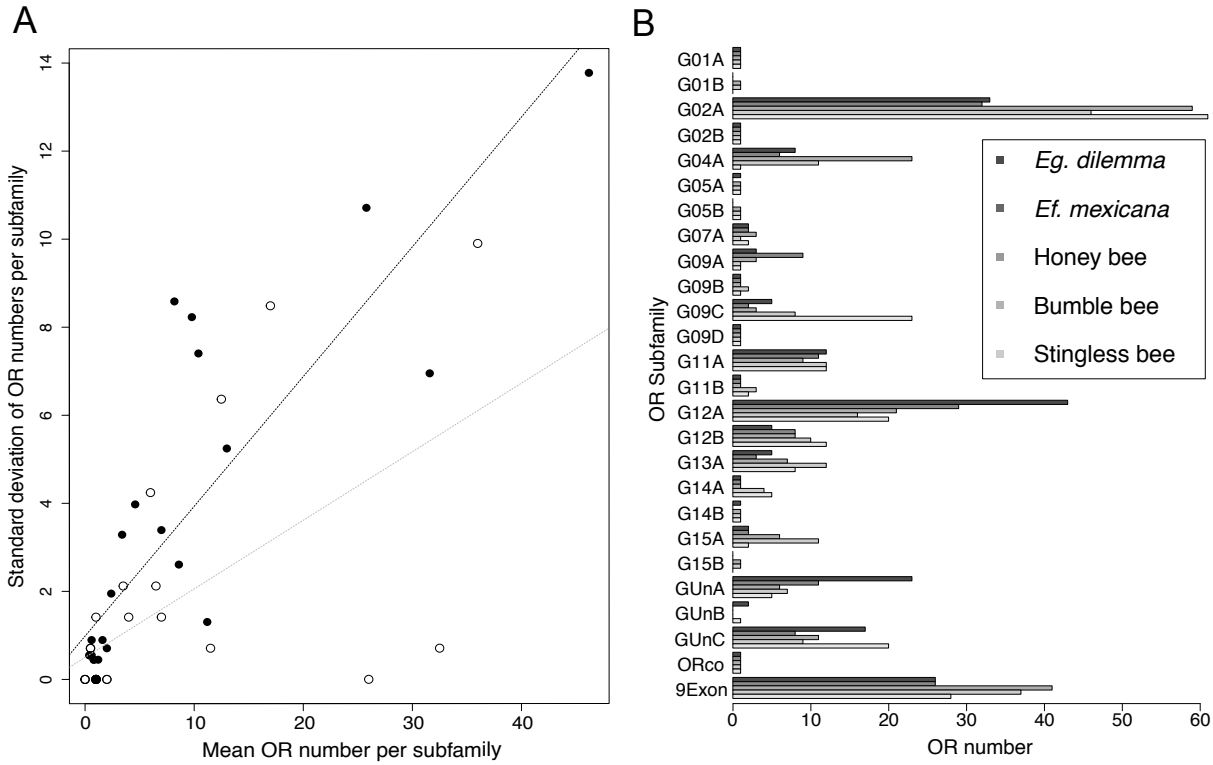


899
900
901

Fig. 2


A)
G02A
GUnA*
G09C*
G15A
GUnB
G01A
G04A
ORco
G07A
G11A
G13A*
'9-exon'
G15B
G01B
G09D
G12B
G11B*
G05B
G05A
G14B
G09A
G02B
G14A
G09B
Bootstrap Support
≥95%
>70%
G12A
GUnC*
B) Subfamily G13A
C) Subfamily G04A
Ef. mexicana
Eg. imperialis
Eg. flammea
Eg. viridissima
Eg. dilemma
El. meriana
El. bombiformis
M. quadrifasciata
B. terrestris
A. mellifera
Branches under positive selection

905 **Fig. 3**



906
907
908
909 **Tables**
910
911 **Tab. 1** Numbers of branches under positive selection for each chemosensory gene
912 family among the four corbiculate bee tribes.
913
914 **Tab. 1**

		Orchid bees	Honey bees	Bumble bees	Stingless bees	Total
ORs	Tested	1234	216	194	237	1881
	P ≤ 0.05	42 (3.4%)	5 (2.3%)	3 (1.6%)	14 (5.9%)	64 (3.4%)
GRs	Tested	116	11	32	15	174
	P ≤ 0.05	17 (14.7%)	2 (18.2%)	5 (15.6%)	4 (26.7%)	28 (16.1%)
IRs	Tested	101	9	9	9	128
	P ≤ 0.05	5 (5.0%)	0 (0%)	0 (0%)	0 (0%)	5 (3.9%)
OBPs	Tested	178	29	18	12	237
	P ≤ 0.05	3 (1.7%)	4 (13.8%)	0 (0%)	0 (0%)	7 (3.0%)
CSPs	Tested	76	6	5	6	93
	P ≤ 0.05	1 (1.3%)	0 (0%)	0 (0%)	0 (0%)	1 (1.1%)

915 All branches tested as well as the numbers and percentages of branches under positive selection after 5% FDR
916 correction are represented.