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Research paper

Patterns of molecular evolution of RNAi genes in social and socially parasitic bumblebees

Sophie Helbing [a], H. Michael G. Lattorff [a,b,*]

[a] Institute of Biology, Molecular Ecology, Martin-Luther University Halle-Wittenberg, Hoher Weg 4, 06099 Halle (Saale), Germany
[b] German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany

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ABSTRACT

The high frequency of interactions amongst closely related individuals in social insect colonies enhances pathogen transmission. Group-mediated behavior supporting immune defenses tends to decrease selection acting on immune genes. Along with low effective population sizes this might result in relaxed constraint and rapid evolution of immune system genes. Here, we show that antiviral siRNA genes show high rates of molecular evolution with *argonaute 2*, *armitage* and *maelstrom* evolving faster in social bumblebees compared to their socially parasitic cuckoo bumblebees that lack a worker caste. RNAi genes show frequent positive selection at the codon level additionally supported by the occurrence of parallel evolution. Their evolutionary rate is linked to their pathway specific position with genes directly interacting with viruses showing the highest rates of molecular evolution. We suggest that higher pathogen load in social insects indeed drives the molecular evolution of immune genes including antiviral siRNA, if not compensated by behavior.

1. Introduction

The success of social insects is mainly attributed to the division of labor amongst workers, but also to the reproductive division of labor between castes with queens monopolizing reproduction whereas workers remain functionally sterile (Lattorff and Moritz, 2013). This reproductive division of labor is enhanced by the high degree of relatedness, allowing kin selection (Hamilton, 1964) to work efficiently.

However, this system may also show some major drawbacks. Social insects are expected to be prime targets for parasites and pathogens (Schmid-Hempel, 1998), as their transmission is enhanced due to frequent social interactions and their establishment might be augmented by the high relatedness within colonies as well as by the high degree of nest homeostasis (Boomsma et al., 2005). Despite the potentially high parasite pressure, a reduced number of innate immune system genes have been recognized within all completely sequenced social insect genomes compared to other, non-social insect species (Evans et al., 2006; Smith et al., 2011; Barribeau et al., 2015 amongst others). Several explanations have been put forward in order to explain this discrepancy. The most prominent one explains the reduced number of immune genes by an advanced level of socially-mediated physiological and behavioral adaptations directed against intruding parasites, summarized as 'social immunity' (Cremer et al., 2007). Indeed, a huge range of activities has been described that altogether might reduce the selection coefficient acting on genes of the innate immune system. In combination with drastically lowered effective population sizes (N_e) in social insects due to the reproductive division of labor (Romiguier et al., 2014), selection efficacy is reduced (Lynch and Conery, 2003). Finally, this will result in non-efficient removal of slightly deleterious mutations (also known as relaxed constraint) that in turn will behave like nearly neutral mutations (Ohta, 1987) so that non-synonymous mutations will increase in frequency and contribute to an increase of non-synonymous divergence between species. The latter one has been frequently observed for social insects implying faster evolution of immune genes (Harpur and Zayed, 2013), especially when compared to solitary insects like *Drosophila* (Viljakainen et al., 2009).

Nevertheless, comparisons between highly eusocial ants or the honeybee (*Apis mellifera*) with solitary insects (e.g., *Drosophila* or *Anopheles*) suffer from several confounding factors. Hymenopteran social insects are haplo-diploid (females are diploid whereas males are haploid), a genetic constitution that by itself reduces N_e (Crozier, 1977). Other factors potentially influencing the rate of molecular evolution might be a higher mutation rate (Schmitz and Moritz, 1998, Bromham and Leys, 2005) and a higher recombination rate (Wilfert et al., 2007) in social insects. Furthermore, these lineages might have had a common ancestor 300 million years ago and might also show lineage specific effects.

Here, we studied the effect of sociality on the rates of evolution of antiviral RNAi genes in a superior system consisting of social bumblebees and their socially parasitic cuckoo bumblebees, both originating from the genus *Bombus* (Cameron et al., 2007). Social species are characterized by annual colonies headed by one single-mated queen

* Corresponding author at: German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany.
E-mail address: lattorff@zoologie.uni-halle.de (H.M.G. Lattorff).

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accompanied by a sterile worker caste ensuring colony growth via division of labor (Goulson, 2010). In contrast, cuckoo bumblebees are non-social due to the lack of a worker caste. The cuckoo bumblebee females enter a host nest during early development of the social nest and kill the resident queen in order to lay their own eggs that will be cared for by the resident host worker force. Cuckoo bumblebees will show an even more reduced N_e than their hosts, as they never may parasitize all available nests (Erler and Lattorff, 2010). This might result in even stronger non-synonymous divergence under relaxed constraint than it might be observed for social species. In a large comparison of several host/social parasite couples spread throughout the order of insects, Bromham and Leys (2005) have shown that indeed, social parasites show higher rates of molecular evolution of a diverse set of DNA sequences (e.g. mitochondrial, nuclear), at least in seven out of eight species pairs.

In this study system we are able to exclude a range of confounding factors previous studies suffered from. Due to their close phylogenetic relationship, social and non-social bumblebees might show less extensive lineage specific effects. Associated characters that might influence the rate of molecular evolution are the same or very similar (haplodiploidy, habitat and environment and the range of pathogens they are exposed to). However, two main characteristics differ as already pointed out. Sociality is associated with higher N_e (Romiguier et al., 2014) as well as with higher recombination rates (Wilfert et al., 2007), both allowing selection to work efficiently. However, as sociality might particularly affect the evolution of immune system genes, we studied the molecular evolution of antiviral genes with respect to life history. Viruses are known to be widespread in social insects (Singh et al., 2010; Levitt et al., 2013; Fürst et al., 2014) and viral replication and mutation rates impose severe requirements on the host's immune-system resulting in an evolutionary arms-race between components of the RNAi-pathway and viruses (Moissiard and Voinnet, 2004; Obbard et al., 2006; Obbard et al., 2009a). Antiviral defense mechanisms are activated in the presence of viral double-stranded (ds)-RNA, leading to the generation of small interfering RNAs (siRNAs) serving as template for RNA-induced silencing complex (RISC)-mediated viral mRNA degradation (Meister and Tuschl, 2004; Buchon and Vaury, 2006; Ding, 2010; Nayak et al., 2013). Counteracting this, viruses encode essential virulence factors, called viral suppressors of RNAi (VSRs) interfering with key-steps of host immune responses (Li and Ding, 2006). Concluding from this, selection pressure on antiviral defense mechanisms might be exceptionally strong.

Cuckoo bumblebees and their respective host species are assumed to be exposed to similar pathogens (e.g. viruses), as they show similarities in their life-cycles and use a shared environment. Hence, they provide an ideal system to study molecular evolution of immune system genes with respect to the effect of sociality. We sequenced six genes of the siRNA pathway from six pairs of social and socially parasitic bumblebee species in order to test for the effect of sociality on the molecular evolution of this pathway. We expect a preponderance of positive selection for these genes resulting in faster evolution of RNAi genes indicated by high divergence due to increased selection pressure induced by pathogens in social species. Under reduced selection pressure, we expect effects of genetic drift to dominate resulting in higher divergence rates for socially parasitic species.

2. Materials and methods

Six genes of the siRNA pathway were partially sequenced and compared across six social *Bombus* species and six of their respective socially parasitic cuckoo bumblebees (*Bombus*, subgenus *Psithyrus*), with each species represented by a maximum of four haploid drones. Detailed information concerning the studied species and their geographic origin is given in Table S1 while the method for DNA extraction has been published elsewhere (Erler et al., 2014).

In order to estimate intra-specific polymorphism 20 and 19 haploid males of *Bombus terrestris* and its social parasite *Bombus vestalis*, respectively, as well as 5 diploid females of *Bombus lapidarius* (workers) and its social parasite *Bombus rupestris* (females) (Table S2) were sequenced for one of the siRNA genes (*r2d2*) and the control gene *IER3* (*immediate early response 3*) using the Ion Torrent PGM (Life Technologies). Samples were collected in 2011 in the Botanical Garden of the Martin-Luther-Universität Halle-Wittenberg (N 51.49, E 11.96) (*B. terrestris*/*B. vestalis*) and in Sangerhausen (N 51.45, E 11.32) (*B. lapidarius*/*B. rupestris*). All individuals were unrelated to each other as revealed by microsatellite genotyping (data not shown).

2.1. Homology searches & primer design

RNAi genes were identified in the draft assembly of the *B. terrestris* (assembly: Bter 1.0, AELG01000001:AELG01010672) and *Bombus impatiens* genome (assembly: Bimp 1.0, AEQM01000001:AEQM01091524) (Sadd et al., 2015) using homology searches based on *Drosophila melanogaster* protein sequences and *A. mellifera* DNA sequences (Honey Bee Genome, Assembly 4.5). Primers were designed on the consensus sequence resulting from the alignment of the *B. terrestris* and *B. impatiens* sequences using Primer3 (Rozen and Skaletsky, 2000). Primers used in this study are reported in Table S3.

2.2. Gene amplification and sequencing

PCR-protocols were chosen according to the fragment length of the target gene. Fragments with expected sizes above 2000 bp were amplified with long-range PCR utilizing TaKaRa LA Taq Polymerase (MoBiTec, Göttingen, Germany). Smaller fragments were amplified using peqGOLD Taq-DNA-Polymerase (peqlab, Erlangen, Germany). PCR protocols are given in **Table** S4. PCR products (except for *dcr2*) were purified using the SureClean PCR purification kit (Bioline, London, UK). *Dcr2* PCR products were gel-purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Sequencing in both directions of *r2d2* (interspecific variation, 12 species), *vig* and *mael* was performed by LGC Genomics (Berlin, Germany) using traditional Sanger sequencing. Sequence chromatograms were inspected and confirmed manually. Forward and reverse sequences were assembled using ContigExpress implemented in Vector NTI Advance 10.2.0 (Invitrogen, Karlsruhe, Germany) in order to detect PCR or sequencing errors.

PCR products of *ago2*, *armi* and *dcr2* were sequenced using the Ion Torrent PGM (Life Technologies). Each species was tagged with a barcode adapter and for each gene all individuals per species were pooled in equimolar amounts. Finally, all amplicons per species were pooled together in equimolar amounts. The barcoded libraries were prepared using the Ion Xpress Barcode Adapters 1-16 Kit following the instructions of the Ion Xpress Plus gDNA and Amplicon Library Preparation user guide for 100 bp sequencing reactions. CLC Genomics Workbench 5.0 (Qiagen) was used to (I) map the reads to the *B. terrestris* reference sequences using default settings and (II) to detect SNP's in the high-throughput sequencing data using the SNP detection tool. The latter was performed using default settings except for the minimum coverage and minimum variant frequency which were adjusted to the library (species/gene) specific requirements.

Sequencing of *r2d2* and *IER3* (intraspecific variation, 4 species) was done as describe above, using the Ion Torrent PGM. Pooled sequences were mapped to reference sequences for the respective species derived from Sanger sequencing. SNPs were detected using the quality-based variant detection function implemented in CLC Genomics Workbench v7.0.4 (Qiagen) using the default settings except for the minimum frequency of variants, which was adjusted to the number of chromosomes and the average coverage.

For eleven species sequences of four control genes without an involvement into immunity responses (*EF-1 alpha*, *Arginine kinase*-AK2, *rhodopsin*, *PEPCK*) published in Cameron et al., 2007 were used, whereas *Bombus perezi* sequences for the same genes (GenBank accession