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# Rapid evolution of antimicrobial peptide genes in an insect host–social parasite system

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ABSTRACT

Selection, as a major driver for evolution in host–parasite interactions, may act on two levels; the virulence of the pathogen, and the hosts' defence system. Effectors of the host defence system might evolve faster than other genes e.g. those involved in adaptation to changes in life history or environmental fluctuations. Host–parasite interactions at the level of hosts and their specific social parasites, present a special setting for evolutionarily driven selection, as both share the same environmental conditions and pathogen pressures.

Here, we study the evolution of antimicrobial peptide (AMP) genes, in six host bumblebee and their socially parasitic cuckoo bumblebee species. The selected AMP genes evolved much faster than non-immune genes, but only *defensin-1* showed significant differences between host and social parasite. Nucleotide diversity and codon-by-codon analyses confirmed that purifying selection is the main selective force acting on bumblebee defence genes.



## 1. Introduction

Host–parasite interactions are shaped by a wide range of biotic and abiotic factors. Under natural conditions, variations in parasite (pathogen) load and virulence, as well as variations in host susceptibility and immune responses, control host–parasite dynamics (Anderson and May, 1982; Gandon et al., 2001; Schmid-Hempel and Ebert, 2003). Co-evolutionary arms races are mostly characterized as reciprocal processes of adaptation and counter-adaptation between parasites and hosts (Dawkins and Krebs, 1979). Positive selection of the defence mechanisms clearly reflects the importance of pathogenic organisms in host evolution, because immunity related proteins are functionally important in the host immune system and play an important role in adapting to novel pathogens or pathogen genotypes.

Parallel evolution at the level of amino acid changes are characterized by parallel replacements between at least two related but distinct species possessing a common ancestor. Convergent evolution describes the same amino acid replacement with the same outcome, in two unrelated species without any common ancestor (Nei and Kumar, 2000). Both, parallel and convergent changes in amino acids are a sign for strong positive selection. However, under natural conditions parallel and convergent evolution have been very rarely observed (Doolittle, 1994).

Evolutionary forces acting on DNA can be characterized by measurable changes of synonymous ($d_S$) and non-synonymous ($d_N$) substitution rates in coding regions (Nielsen, 2005; Yang and Bielawski, 2000). The $d_N/d_S$ ratio ($\omega$) classifies possible occurring selection events in three different groups: $\omega > 1$, diversifying selection (positive selection); $\omega = 1$, no selection (neutral evolution) and $\omega < 1$, purifying selection (negative selection) (reviewed in Wagner, 2002).

Innate insect immune systems categorise the majority of parasites into four groups: viruses, gram-positive and gram-negative bacteria, and fungi or yeasts (Hultmark, 1993; Lemaitre and Hoffmann, 2007). Selection may take place separately at genes specific for each group, or globally against one of the groups. Social insects are model organisms for investigating adaptive evolution in the innate immune system, as group living increases their vulnerability to diseases, especially since the group is composed of closely related individuals (Schmid-Hempel, 1998).

Less attention has been paid to host–parasite systems where both host and parasite are closely related, sharing similar life his-

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tory traits. This is known as social parasitism or brood parasitism and is found in both birds and social insects (e.g. ants and bumblebees, reviewed in Kilner and Langmore, 2011). Host bumblebees and their social parasites, cuckoo bumblebees (Fisher, 1988; van Honk et al., 1981) share the same, but timeshifted annual life cycle, and corresponding environmental conditions (Alford, 1975; Sladen, 1912). Host bumblebee colonies are comprised of drones (males), workers and a single bumblebee queen who initiates nest foundation in early spring. During colony development, workers are produced exclusively and only at the end of the season are new sexuals (drones and queens) produced for the forthcoming season (Alford, 1975; Sladen, 1912). Cuckoo bumblebee females invade host nests in spring, killing the host queen and leaving host workers to take care of the cuckoo female's brood. In contrast to the host, cuckoo females produce only male and queen offspring, lacking a worker caste (Alford, 1975; Sladen, 1912). This kind of parasitism is assigned to queen-intolerant inquilines (reviewed in Brandt et al., 2005). Within this asymmetric, inter-specific arms race (Dawkins and Krebs, 1979), cuckoo bumblebees may be specialist or generalist, being a mono- or multiple-host social parasite, depending on the host species range (Loken, 1984).

Multiple reports of plant and animal evolutionary adaptations suggest that the environment plays an important role in gene evolution and associated phenotypic shifts (reviewed in Levasseur et al., 2007; Salamin et al., 2010). Sharing the same environmental conditions (i.e. food source, homeostatic nest condition and symbionts, non-coevolving saprophytes, omnipresent microorganisms at nest and hibernation sites), including co-evolving parasites and pathogens, might force parallel evolution of parasite/pathogen defence mechanisms against common microbes and viruses, in bumblebee hosts and their cuckoo bee parasites. Environmental conditions (e.g., temperature, humidity and light) and shifts in the microhabitat or diets show a strong impact (37.5%; Fuller et al., 2011) on immunocompetence and pathogen susceptibility in social insects (Bulmer and Crozier, 2006; Fuller et al., 2011). Transitions to new habitats represent the exposure of the host to novel pathogens, which could direct rapid, adaptive changes in immune proteins (Bulmer and Crozier, 2006). As a sign of adaptive evolution, genes involved in the immune defences of various plants and animals, typically show a faster rate of nucleotide and amino acid substitutions (non-synonymous), than non-immunity related genes (McTaggart et al., 2012; Obbard et al., 2009; Sackton et al., 2007; Tiffin and Moeller, 2006; Trowsdale and Parham, 2004; reviewed in Bulmer, 2010).

Positive selection and rapid gene duplication as factors influencing evolution have been demonstrated in social insects for antimicrobial peptides (AMPs) (termicin – Bulmer and Crozier, 2004), gram-negative bacteria-binding proteins and relish in termites (Bulmer and Crozier, 2006) and several immune genes in ants (Viljakainen and Pamilo, 2008; Viljakainen et al., 2009). Positive selection was detected mostly in the mature region of the AMPs, whereas the signal and pro-regions seem to evolve neutrally (Lazzaro and Clark, 2003; Viljakainen and Pamilo, 2008). For termicin especially, the expressed mature peptide appears to have diverged more rapidly than the 3′UTR (Bulmer and Crozier, 2004). In addition, a population genetic analysis of nucleotide intra-specific polymorphism and inter-specific divergence indicated that a positive selection driven selective sweep reduced polymorphisms in the AMP termicin (Bulmer et al., 2010). Hence, if the immune system adapts to parasites/pathogens in similar ways in related species (i.e. host and cuckoo bumblebee species), we would expect to observe congeneric genes experiencing positive selection in different lineages of the same affiliation.

Social insects show a reduced number of immune genes relative to solitary species (Evans et al., 2006), and so may compensate for the reduction in immunity gene variance through group level 'social immunity' (Cremer et al., 2007; Richter et al., 2012; Traniello et al., 2002).

Social parasites and their hosts are frequently very close phylogenetic relatives that might influence the ease of evolutionary adaptations between host and social parasite on both sides (Davies et al., 1989). Here we tested whether parasite or pathogen driven evolutionary adaptations (parallel evolution of AMP genes) can be observed in closely related host-social parasite couples sharing the same environmental conditions i.e. parasite pressure (Erler et al., 2012). Six specialised host/social parasite bumblebee couples were used to determine the type and strength of selection on AMP genes, both within and between host and social parasite species.

## 2. Material and methods

### 2.1. Bumblebee samples

Bumblebee drones of six bumblebee hosts and their respective cuckoo bumblebee species were sampled in three locations across Europe (Table 1). Bumblebees have a haplo-diploid sex determination system; therefore the haploid drones provide a highly efficient model system for genetic studies as they present a single allele per locus. At each location, host and social parasite couples were caught during foraging flights and immediately stored in ethanol or at −80 °C until further processing. Bumblebee species were identified using the taxonomic key of Mauss (1994).

### 2.2. DNA isolation and target gene amplification

The thorax muscles of three individuals per species were used to isolate genomic DNA using the DNeasy Blood & Tissue Kit tissue protocol (Qiagen, Hilden, Germany). Tissue samples were homogenized, followed by proteinase K (600 mAU/mL) treatment for at least 2 h and final DNA elution was conducted twice in 50 μL AE Buffer. Quality and quantity of DNA was determined via NanoDrop ND-1000 (Peqlab, Erlangen, Germany).

AMP (*abaecin*, *defensin-1* and *hymenoptaecin*; all bumblebees) and non-immune reference gene – (*EF-1 alpha*, *arginine kinase*, *rhodopsin*, *PEPCK*; only for *B. perezi*) amplification was performed in a thermocycler, with denaturation at 95 °C for 4 min; 35 cycles at 95 °C for 40 s; 55 °C for 30 s, and 72 °C for 2 min 20 s, with final elongation at 72 °C for 10 min. Each reaction (10 μL) contained 2.0 mM dNTPs, 0.2 μM of each gene-specific forward and reverse primer (Table 2), 0.25 U of peqGOLD Taq-DNA-polymerase (Peqlab, Erlangen, Germany) and 1 μL of extracted genomic DNA.

PCR products were checked for correct amplicon size by automated multicapillary electrophoresis using the QIAxcel System with QIAxcel DNA High Resolution Kit (Qiagen, Hilden, Germany), purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or SureClean (Bioline, Luckenwalde, Germany), before 96-well plate MTP sequencing by LGC Genomics (Berlin, Germany), based on traditional Sanger sequencing. All fragments were sequenced overlapping in both directions. *Abaecin*, *defensin-1* and *hymenoptaecin* were successfully amplified in all 12 host and social parasite bumblebee species listed in Table 1. The sequenced regions did not cover the entire coding region of the genes but lacked a few nucleotides of the coding regions in either the 3′ or 5′ end, or both.

A sequenced region of the 16S rRNA was used to confirm species identification of the cuckoo bumblebee species by comparison with reference sequences from GenBank (Cameron et al., 2007).

AMP gene sequences for all bumblebee species, non-immune and 16S rRNA gene sequences for *B. perezi* are available on GenBank, under the accession numbers: KC662127-38 (*abaecin*),

**Table 1**
Bumblebee host and social parasite species overview, including sampling location and year.

| | Species (subgenera) | Location | WGS coordinates | Country, region | Year |
|---|---|---|---|---|---|
| Host | *B.* (*Bombus*) *lucorum* | Nyer | 42°30′N 2°19′E | France, Pyrenees | 2007 |
| Parasite | *B.* (*Psithyrus*) *bohemicus* | Nyer | 42°31′N 2°17′E | France, Pyrenees | 2007 |
| Host | *B.* (*Bombus*) *terrestris xanthopus*[a] | Morosaglia | 42°15′N 9°11′E | France, Corsica | 2007 |
| Parasite | *B.* (*Psithyrus*) *perezi*[a] | Morosaglia | 42°24′N 9°11′E | France, Corsica | 2007 |
| Host | *B.* (*Megabombus*) *ruderatus corsicola* | Guaitella | 42°25′N 9°25′E | France, Corsica | 2008 |
| Parasite | *B.* (*Psithyrus*) *maxillosus italicus*[b] | Haut-Asco | 42°14′N 8°33′E | France, Corsica | 2008 |
| Host | *B.* (*Bombus*) *terrestris terrestris* | Halle (Saale) | 51°29′N 11°58′E | Germany, Saxony-Anhalt | 2009 |
| Parasite | *B.* (*Psithyrus*) *vestalis* | Halle (Saale) | 51°29′N 11°58′E | Germany, Saxony-Anhalt | 2009 |
| Host | *B.* (*Melanobombus*) *lapidarius* | Halle (Saale) | 51°29′N 11°58′E | Germany, Saxony-Anhalt | 2009 |
| Parasite | *B.* (*Psithyrus*) *rupestris* | Halle (Saale) | 51°29′N 11°58′E | Germany, Saxony-Anhalt | 2009 |
| Host | *B.* (*Thoracobombus*) *pascuorum* | Halle (Saale) | 51°29′N 11°56′E | Germany, Saxony-Anhalt | 2009 |
| Parasite | *B.* (*Psithyrus*) *campestris* | Halle (Saale) | 51°29′N 11°56′E | Germany, Saxony-Anhalt | 2009 |

[a] according to Lecocq et al. (2013).
[b] recently renamed to *Bombus barbutellus maxillosus* (Lecocq et al. (2011).

**Table 2**
Primer information for the immune and non-immune genes.

| Gene | Primer | Sequence (5′–3′) | $T_m$ | Reference |
|---|---|---|---|---|
| *Abaecin* | A1-F | CCGCCACGACCGGGACAATC | 67 | This study |
| | A1-R | GAAACGAAACCGCGTGCGAA | 60 | This study |
| *Defensin-1* | def_1529-F | AAACGCAGAAAGACAAAACG | 54 | This study |
| | def_2933-R | CGAAACGTTTGTCCCAGAG | 57 | This study |
| *Hymenoptaecin* | H-F | ACTGGCTCTCTTCTGCATGG | 60 | This study |
| | H-R | GAAGCTGGGCGAGATTCTG | 59 | This study |
| *Arginine kinase* | ArgK2-F | GACAGCAARTCTCTGCTGAAGAA | 62 | [a] |
| | ArgK2-R | GGTYTTGGCATCGTTGTGGTAGATAC | 67 | [a] |
| *EF-1 alpha* | F2-ForH | GGRCAYAGAGATTTCATCAAGAAC | 62 | [b] |
| | F2-RevH2 | TTGCAAAGCTTCRKGATGCATTT | 59 | [b] |
| *PEPCK* | FHv4 | TGTATRATAATTCGCAAYTTCAC | 56 | [c] |
| | RHv | CTGCTGGRGTYCTAGATCC | 59 | [c] |
| *Rhodopsin* | LWRhF | AATTGCTATTAYGARACNTGGGT | 58 | [d] |
| | LWRhR | ATATGGAGTCCANGCCATRAACCA | 64 | [d] |
| 16S rRNA | 16SWb | CACCTGTTTATCAAAAACAT | 50 | [e] |
| | 874-16SIR | TATAGATAGAAACCAATCTG | 50 | [f] |

$T_m$, melting temperature.
[a] Kawakita et al. (2003).
[b] Hines et al. (2006).
[c] Cameron et al. (2007).
[d] Mardulyn and Cameron (1999).
[e] Dowton and Austin (1994).
[f] Cameron et al. (1992).

KC662139-50 (*defensin-1*), KC662151-62 (*hymenoptaecin*), KC662163-67 (*B. perezi* genes).

### 2.3. Sequence analysis and test for selection in AMP genes

ContigExpress and AlignX, implemented in Vector NTI Advance 10.3.0 (Invitrogen, Carlsbad, CA, USA), were used for sequence alignments, visual inspection and manual editing of the sequences. Exon–intron structure of the AMP genes was verified by sequence comparison with AMP gene sequences of *B. t. terrestris* from GenBank (XM_003394654, XM_003395924, XR_132450) and Erler et al. (2011), and of *B. impatiens* (GenBank: XM_003491496, XM_003486302, XM_003494885).

DnaSP v5.10.1 (Librado and Rozas, 2009) was used to calculate nucleotide diversity (synonymous and non-synonymous sites, including Jukes-Cantor correction (Jukes and Cantor, 1969)) for *defensin-1*, *hymenoptaecin* and non-immune genes (GenBank accession numbers see Cameron et al., 2007) over all species, for the host species only, social parasite species only and for all pair-wise comparisons within hosts and social parasites. A Mann–Whitney *U* (MWU) test, implemented in STATISTICA 8.0 (StatSoft, Tulsa, OK), was used to test for significant differences in nucleotide diversity

between AMP and non-immune genes and between host and social parasite bumblebee species using all pair-wise comparisons within hosts and within social parasites.

Codon-by-codon analyses of evolutionary changes reveal codon specific adaptations, which indicate either parallel or convergent evolution of AMP genes. Two different methods were applied to test both scenarios (reviewed in Anisimova and Liberles, 2007).

(1) Selecton version 2.4 (Stern et al., 2007) calculates nucleotide changes of each codon to detect positive and purifying selection using a Bayesian inference approach. Default settings for 'Positive selection enabled (M8, beta + w > = 1)' were used, including *B. t. terrestris* as the template sequence and a reference phylogenetic tree as the initial tree (for details see 2). Selecton infers site-specific *Ka*/*Ks* (also known as $d_N/d_S$) by computing the expectation of the posterior distribution at each site. After determining the probability for positive selection, the assumption of positive selection will be compared to the null model: no positive selection (M8a, beta + w = 1), using a likelihood ratio test (LRT) (Yang et al., 2000). If there is no significant difference between M8 and M8a, the hypothesis of positive selection can be rejected and no codon shows any kind of positively selected sites.
(2) Determination of the ancestral state for each codon position (Nei and Kumar, 2000, Chapter 11.4): If several bumblebee species might share the same codon changes from the same ancestral state, such changes will hint towards parallel evolution. The reconstruction of the ancestral state for codons of AMP genes is done according to a reference phylogenetic tree based on the non-immune gene set. The genes *EF-1 alpha*, *arginine kinase*, *rhodopsin* and *PEPCK* (Cameron et al., 2007) were used to reconstruct the reference host-social parasite phylogeny. Model selection (tree to use: neighbor-joining tree, statistical method: Maximum Likelihood, substitution type: amino acid), implemented in MEGA v5.1 (Tamura et al., 2011) was used to determine the best model to reconstruct the bumblebee phylogeny from this data set. For both data sets (AMP and non-immune genes) the Jones–Taylor–Thornton (JTT) model, including bootstrap method with 500 replicates, was selected to be the best substitution model. Finally, the ancestral state was estimated using the non-immune gene tree (user tree) as a template for the AMP gene tree. A phylogenetic tree was established including all possible changes at each ancestral state and a list of amino acid changes along the target sequence. Comparing amino acid changes over host and social parasite couples, or only within the group host and within the group social parasite, shows candidates for selection and parallel evolution. A similar function for the analysis of the ancestral state is implemented in Selecton v2.4 and was used to double-check the results from MEGA analysis.

Classical phylogenetic reconstruction based on nucleotide substitutions was used to check for the overall pattern of changes in the AMP gene coding sequences and clustering of host and social parasite species compared to the reference tree (non-immune genes). Model selection revealed the Kimura 2-parameter model, which considers transitional and transversional substitution rates, as the best model. Bootstrap resampling method (1000 replications) was used to verify the topology of the inferred phylogenetic tree.

In addition, to check if evolutionary changes might also occur in non-coding AMP sequences, we analyzed the phylogenetic relationships of the six host and social parasite bumblebee couples using Maximum Likelihood analysis of the genetic information from all three AMP genes (*abaecin*, *defensin-1*, *hymenoptaecin*). Different models were used for: (1) all coding and non-coding regions (model: Tamura 3-Parameter including gamma distribution), (2) only coding regions (model: Kimura 2-Parameter) and (3) only non-coding regions (model: Tamura 3-Parameter), including bootstrap testing of the robustness of the final tree (1000 replications). Different models were selected on the lowest BIC (Bayesian Information Criterion) score using Model selection implemented in MEGA v5.1 (Tamura et al., 2011).

## 3. Results

### 3.1. Evolutionary speed of AMP genes

The sequenced regions did not cover the entire coding region of the genes, lacking a few coding regions in either the 3′ or 5′ end, or both. In detail, 33 bp of the *abaecin* coding region and up to 965 bp non-coding sequences were successfully amplified for all bumblebee species. *B. pascuorum* (893 bp), followed by *B. ruderatus* (946 bp), showed the lowest nucleotide number for the non-coding sequence due to many species specific deletions. An intron yet to be described was detected in all bumblebee species, located between amino acid seven and eight, with characteristic acceptor and donor splice sites GT/AG. For *defensin-1*, 60 bp of the pro region and 120 bp of the mature peptide coding region were used for analysis and up to 1031 bp non-coding sequences (*B. campestris* showed the lowest number – 418 bp). Two bumblebee species had large non-coding region sequence gaps for *defensin-1* (*B. m. italicus*: ca. 180 bp, *B. perezi*: ca. 600 bp) due to unsatisfactory sequencing results. Finally, 21 bp of the pro region and 210 bp of the *hymenoptaecin* mature peptide sequence were sequenced, including up to 900 bp non-coding sequence. Once again, *B. campestris* had the shortest non-coding sequence (432 bp) because of many species specific deletions, followed by *B. lapidarius, B. r. corsicola* and *B. pascuorum*. Details on the pro and mature peptide region and the non-coding introns of each AMP, over all bumblebee species, are visualized in the Supplementary Figs. S1–S3, using nucleotide sequence alignments (using MAFFT version 6; Katoh and Toh, 2010; Katoh et al., 2002) and the AMP annotation of *B. ignitus* (Choi et al., 2008).

The coding regions of all analyzed antimicrobial peptide genes (*abaecin*, *defensin-1, hymenoptaecin*) are highly conserved over the 12 bumblebee species. *Defensin-1* homology values (percent identity at nucleotide level) ranged between 94.35% (between *B. campestris* and the group of *B. t. terrestris, B. t. xanthopus, B. lucorum*) and two groups with 100% (1) *B. t. terrestris, B. t. xanthopus, B. lucorum* and (2) *B. m. italicus, B. perezi, B. vestalis*. *Hymenoptaecin* showed nucleotide conservation within a range of 93.45% (between *B. pascuorum* and the group of *B. t. terrestris, B. t. xanthopus, B. lucorum*) and 100% (between *B. t. terrestris, B. t. xanthopus* and *B. lucorum*). *Abaecin* coding region revealed identity ranges between 93% and 100%, but without any distinct clustering due to the small amount of analyzed nucleotides. Only *B. lapidarius* and *B. r. corsicola* showed sequence differences compared with the others in the *abaecin* coding region.

All tested AMP genes evolved much faster than the non-immune genes, irrespective of species affiliation (overall: MWU, $Z = -13.715$, $N = 366$, $P < 0.001$; only host species: MWU, $Z = -7.015$, $N = 78$, $P < 0.001$; only social parasite species: MWU, $Z = -3.160$, $N = 72$, $P = 0.002$). Substitution rates ($d_N/d_S$) of AMP genes ranged between 0.099–0.672, and 0–0.06 for the non-immune genes (Table 3). When comparing evolutionary changes between host and social parasite bumblebee species, only *defensin-1* showed significant differences (MWU test, $P = 0.01$, Table 3) with host species having evolved much faster than the social parasite species.

**Table 3**
$d_N/d_S$ ratios for host and social parasite immune (*defensin-1*, *hymenoptaecin*) and non-immune genes (*arginine kinase*, *EF-1 alpha*, *PEPCK*, *rhodopsin*). Mann–Whitney *U* (MWU) test shows significant differences between groups ($P < 0.05$).

| Gene | $d_N/d_S$ (Overall) | $d_N/d_S$ (Host) | $d_N/d_S$ (Parasite) | **MWU-Test** (Host *vs.* Parasite) |
|---|---|---|---|---|
| *Defensin-1* | 0.243 | 0.267 | 0.099 | 0.001 |
| *Hymenoptaecin* | 0.367 | 0.225 | 0.672 | 0.085 |
| Mean | 0.305 | 0.246 | 0.386 | |
| *Arginine kinase* | 0.01 | 0.019 | 0 | 0.108 |
| EF-1 alpha | 0.005 | 0.008 | 0 | 0.108 |
| *PEPCK* | 0.017 | 0.043 | 0 | 0.002 |
| *Rhodopsin* | 0.035 | 0.060 | 0 | <0.001 |
| Mean | 0.017 | 0.033 | 0 | |

### 3.2. Non-coding sequences

Comparing nucleotide substitutions of the AMP non-coding sequences (only introns available) revealed no clear signal for either parallel or convergent evolution between hosts and their social parasites for each of the six bumblebee couples. Mostly constant patterns of indels and nucleotide substitutions were observed, demonstrating a similar evolutionary pattern within the group of hosts and social parasites, respectively (see Supplementary Figs. S1–S3). *Abaecin* showed constant patterns of indels and substitutions between *B. lucorum*, *B. t. terrestris*, and *B. t. xanthopus* (including one obvious insertion – GTAT) and between *B. m. italicus*, *B. perezi*, and *B. vestalis*. The same phenomenon was detectable for *defensin-1* and *hymenoptaecin*, showing constant patterns of indels and substitutions between *B. lucorum*, *B. t. terrestris*, *B. t. xanthopus* (e.g. GCGACTATTCG insertion for *defensin-1*). In addition, reconstructing the phylogenetic relationship of the 12 bumblebee species using non-coding sequence substitution patterns revealed an equal separation in two major groups: the first including the social parasites and second the host bumblebee group (Supplementary Fig. S4). Thus, the same pattern was ascertained as in Fig. 1 and also in agreement with the bumblebee phylogeny by Cameron et al. (2007).

### 3.3. Selection and evolution of AMP genes

In order to investigate the role of positive selection on the evolution of AMP genes, a codon-based Bayesian inference approach was applied. *Hymenoptaecin* showed a significant difference when comparing the two models among the 12 different bumblebee species (log-likelihood M8 model = −361.669, M8a model = −365.744; LRT $P = 0.01$) (Fig. 2), which may indicate positive selection acting on *hymenoptaecin*. *Defensin-1* did not pass the level of significance (log-likelihood M8 model = −336.601, M8a model = −337.762; LRT $P > 0.05$). Purifying selected sites were detectable for both *Ka/Ks* scores below 1 (Fig. 2), with a slightly higher amount for *hymenoptaecin* compared to *defensin-1*. Only a minority of all selectable sites (<15%) had an evolutionary speed up to ~5 times than the basic level of the whole gene.

The reconstruction of the ancestral state sequences for *defensin-1* and *hymenoptaecin* did not show any general pattern of parallel evolution between bumblebee host-social parasite couples (Table 4). Mostly *B. rupestris, B. bohemicus* and *B. campestris* – subgenera *Psithyrus* (Hymenoptaecin H8Y) – but without a specific common ancestor (Fig. 1); and *B. t. terrestris, B. t. xanthopus, B. lucorum* – subgenera *Bombus* – build up a separate group by Hymenoptaecin changes Q18D and Q20K, respectively. Synonymous changes along both sequences confirmed this pattern and also include the cluster *B. m. italicus*, *B. perezi* and *B. vestalis* – subgenera *Psithyrus*. Comparing host and social parasite species changes, only codon 10 of *defensin-1* showed an unambiguous separation of the host from the social parasite species by G10A (Table 4). Additional comparison of nucleotide changes along the AMP and non-immune genes

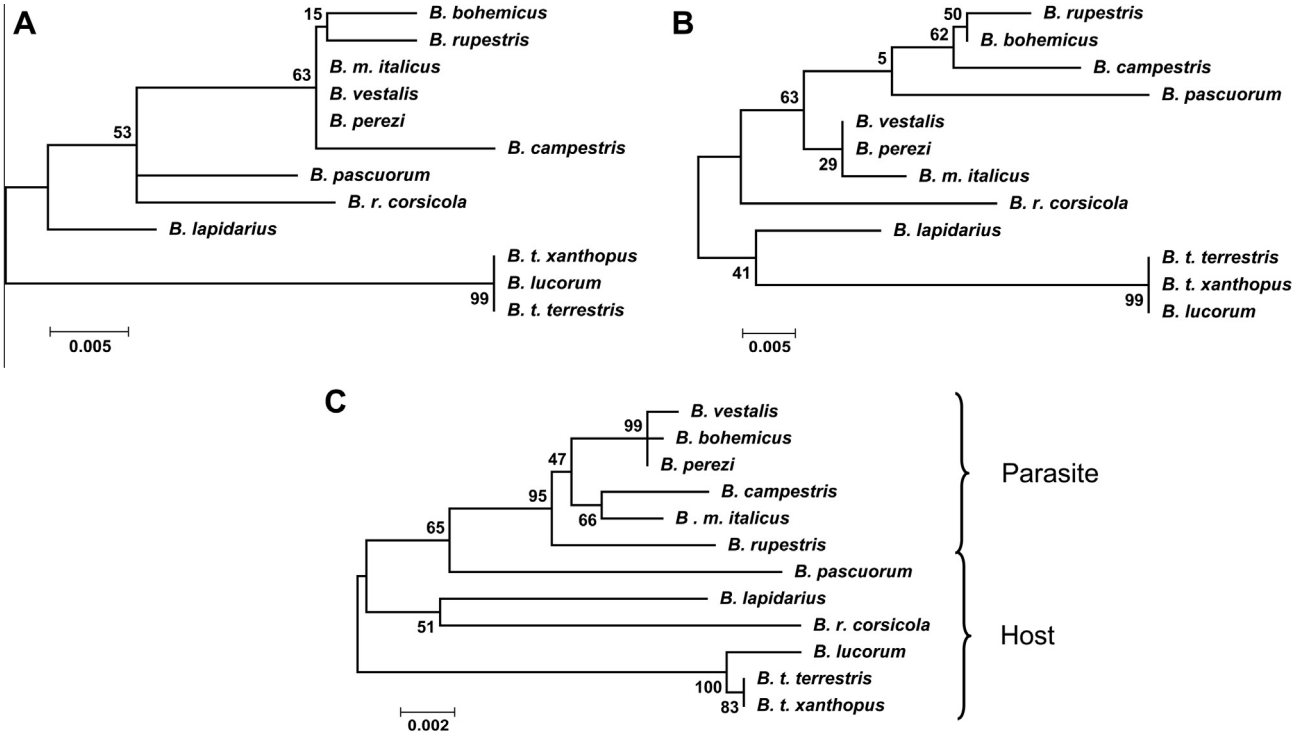


**Fig. 1.** Phylogenetic relationships of the six host and social parasite bumblebee couples for the coding sequences of *defensin-1* (A), *hymenoptaecin* (B) and non-immune reference genes (C). Maximum Likelihood analysis was used with Kimura 2-parameter model method (including test of phylogeny: bootstrap with 1000 replications). Note different scaling between A, B and C.

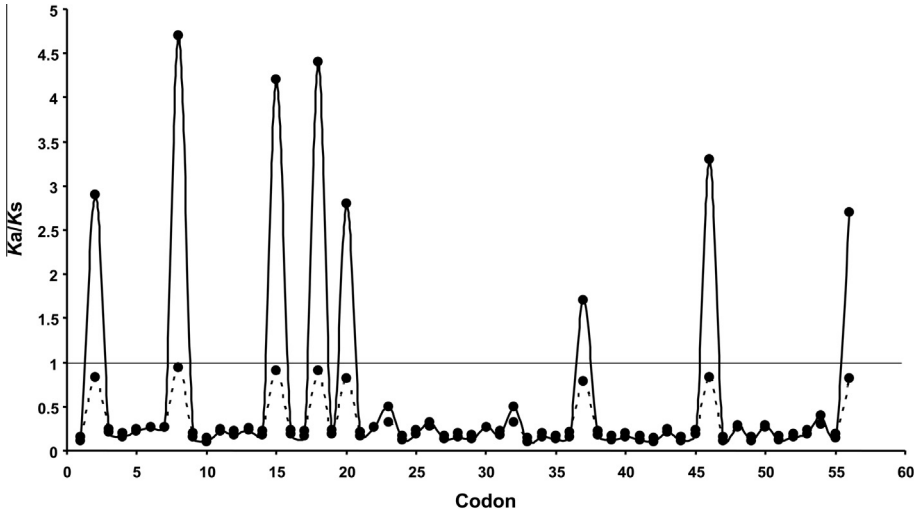


**Fig. 2.** *Ka*/*Ks* scores obtained for each codon position of *hymenoptaecin*. Bayesian models, which assume a statistical distribution to account for heterogeneous *Ka*/*Ks* values among sites, are plotted for each codon (M8 model – solid line, allows for positive selection; M8a model – dashed line, only neutral and purifying selection are allowed). (*Ka*, non-synonymous rate; *Ks*, synonymous rate at each codon site.)

**Table 4**
Amino acid substitutions related to the ancestral state for Defensin-1 and Hymenoptaecin.

| | Defensin-1 | | | | | | Hymenoptaecin | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Position | 5 | 6 | 8 | 10 | 16 | 55 | 2 | 8 | 15 | 18 | 20 | 37 | 46 | 56 |
| Ancestral | P | L | H | G | D | I | S | H | L | Q | Q | H | V | I |
| *B. lucorum* | L | · | Q | · | · | V | · | | · | D | K | · | · | · |
| *B. bohemicus* | · | · | · | A | · | · | · | Y | V | · | · | · | · | · |
| *B. t. xanthopus* | L | · | Q | · | · | V | · | · | · | D | K | · | · | · |
| *B. perezi* | · | · | · | A | · | · | · | · | V | · | · | · | · | · |
| *B. r. corsicola* | · | · | · | D | V | · | · | · | · | · | · | · | · | V |
| *B. m. italicus* | · | · | · | A | · | · | · | · | V | · | · | · | · | · |
| *B. t. terrestris* | L | · | Q | · | · | V | · | · | · | D | K | · | · | · |
| *B. vestalis* | · | · | · | A | · | · | · | · | V | · | · | · | · | · |
| *B. lapidarius* | · | · | · | D | · | V | · | · | · | · | · | · | M | · |
| *B. rupestris* | · | F | · | A | · | · | · | Y | V | · | · | R | · | · |
| *B. pascuorum* | · | · | · | · | · | · | Y | · | V | · | · | · | · | · |
| *B. campestris* | · | · | · | A | · | · | · | Y | I | · | · | · | · | · |

also confirmed the absence of a general sign for parallel evolution between host and social parasite couples – AMP genes evolve in a similar pattern but more within bumblebee lineage or subspecies specific than adapted to environmental changes between the host and its social parasites. Fig. 1 illustrates the evident clustering of host and social parasite species into two separate groups for *defensin-1* and *hymenoptaecin* in comparison to the four non-immune genes. Grouping in host and social parasite group was almost consistent between the three different trees, except for the *hymenoptaecin* tree where *B. pascuorum* was placed within the social parasite section (Fig. 1B), which is not surprisingly as *B. pascuorum* (*Thoracobombus*) is the closest relative of the subgenus *Psithyrus* in our dataset (according to Cameron et al., 2007). However, comparing the bootstrap consensus tree with the original tree in respect of bootstrap values (39 for bootstrap consensus tree vs. 5 for original tree), *B. pascuorum* definitely belongs to the host bumblebee group, consistent with any other tree analyzed in this study (see Fig. 1 and Supplementary Fig. S4). The topology of the AMP gene trees at the level of the subgenera (Fig. 1) was in agreement with the extensive bumblebee phylogeny constructed by Cameron et al. (2007). Both clusters, separated in host and social parasite bumblebees, confirmed the absence of parallel evolution between hosts and social parasites sharing the same environment and indicated a strongly similar evolutionary pattern between closely related subspecies. Furthermore, they cluster in accordance with their phylogenetical information for the major subgenera *Bombus*, *Psithyrus* and *Thoracobombus*. Only *B. lapidarius* and *B. r. corsicola* move from one group to another according to the sequence considered.

## 4. Discussion

### 4.1. Evolutionary speed of AMP genes

Bumblebee substitution rates for AMP genes do not differ from other social insects, such as ants and honey bees (*defensin-1*: 0.243 vs. 0.5 in ants, 0.272 in honey bees; *hymenoptaecin*: 0.367 vs. 0.299 in honey bees and *abaecin*: 0.3 vs. 0.3 in ants – Erler and Lattorff, unpublished data; Viljakainen and Pamilo, 2008), indicating high evolutionary rates at the protein level. The median substitution ratio ($d_N/d_S$) was substantially higher in social insects (bumblebees – 0.305, this study; honey bees – 0.269; ants – 0.500) compared to non-social insects (*Drosophila* – 0.08; *Nasonia* (only *defensin-1* available) – 0.08) (Gao and Zhu, 2010; Sackton et al., 2007; Viljakainen and Pamilo, 2008). Though, substitution ratios of immune genes can be influenced by the group of immune gene (recognition, signalling or effector genes) and the type of outgroup used, (genus or order specific, e.g. *D. simulans*: immunity 0.268, non-immunity 0.082; *D. melanogaster*: immunity 0.207, non-immunity 0.172; outgroup: *D. yakuba*; Schlenke and Begun, 2003). When comparing the

$d_N/d_S$ ratio of non-immune genes, the overall ratio was much lower in bumblebees (0.017) than in honey bees (0.036) and flies (0.045) (Viljakainen and Pamilo, 2008), which may be explained by the low number of non-immune genes.

A McDonald–Kreitman test (McDonald and Kreitman, 1991); which compares non-synonymous and synonymous changes and contrasts within-species polymorphism to fixed differences between species; could not be used to test for within population differences in adaptation due to the limited low sample size for the majority of rare parasitic cuckoo bumblebee species. However it should be noted that estimates of the rate of adaptive substitution can be influenced by factors such as population demography (Hughes, 2007).

### 4.2. Selection and evolution of AMP genes

Immune systems of social insects (e.g. ants, bees and termites) and dipteran insects (e.g. flies and mosquitoes) may respond differently to the selection pressure caused by microbial parasites and pathogens. No evidence for positive selection has been found in antibacterial or antifungal peptide genes of *Drosophila* and *Bombyx mori* (Jiggins and Kim, 2005; Lazzaro and Clark, 2003; Sackton et al., 2007; Yang et al., 2011) and immune genes, including several AMPs of *Anopheles* (Lehmann et al., 2009; Parmakelis et al., 2008; Simard et al., 2007) indicated no sign for co-evolution with parasites and pathogens. Host–parasite arms races may involve strong selection, but only on a relatively small subset of the immune system, such as Imd- and RNAi-pathway genes (Obbard et al., 2009).

Insects might differ in how they respond to parasite pressure, as positive selection acting on AMP genes has so far been found only in social insect species. Further evidence was found in this study, in the form of positive selection acting on *hymenoptaecin*. Yet irrespective of selection pressure, qualities of AMPs influence important host parameters, such as survivorship after bacterial infection (Coggins et al., 2012). As no signal for positive selection has been shown in AMPs for honeybees, except for *defensin-2*, (Harpur and Zayed, 2013) and in only a few ant, bumblebee and termite genes; immune genes may possibly attack a wide spectrum of non-coevolving bacteria (saprophytes), or selection is acting instead for speed and efficiency of AMP production (Sackton et al., 2007; Simard et al., 2007). Positive selection may play a limited role in the evolution of innate immune genes and relaxed purifying selection, including high rates of non-synonymous polymorphisms and divergence act as central mechanism on immune gene evolution (Harpur and Zayed, 2013). Adaptation of AMPs to non-coevolving, universal saprophytes might be the reason for the absence of any parallel substitution pattern related to the couples of bumblebee host and social parasite species. Purifying selection seems to be the common mode of selected changes among AMP genes of insects, as shown for *defensin-1*, regardless of whether they are social or non-social. Certainly, the above mentioned results of ant and termite studies should be treated with caution, as suggested by Hughes (2012), since the statistical methods used are known to have a very high rate of false positives and no experimental evidence has been provided concerning the biological function of selection affecting amino acid replacements.

The major difference between ants, termites and bumblebees is the life cycle itself. Compared with bumblebees, ants and termites have long-lived colonies, with overlapping generations staying in the same nest, which might create a stable and long-lasting association between the host and its specific pathogens. Such an association might be favourable for positive selection on immunity related genes (Viljakainen and Pamilo, 2008). The evidence of positive selection in AMPs might suggest that AMPs in bumblebees are involved in lineage-specific host–parasite arms races; and no evidence was detected that closely related species experiencing comparable selection pressures, adapt to such selection pressures in similar ways.

Several theories were put forward to explain positive selection and AMP gene evolution in social insects and three, mutually non-exclusive hypotheses, have been put forward:

(1) The rate of non-synonymous substitutions might be elevated in social insects not only because of positive selection, but also by low effective population sizes (see Erler and Lattorff, 2010) and nearly neutral mutations might behave as if neutral (Bromham and Leys, 2005). A small effective population size would increase the rate of slightly harmful amino acid substitutions in all genes and raise the $d_N/d_S$ ratios (Bromham and Leys, 2005; Viljakainen et al., 2009).
(2) Whilst purifying selection on innate immunity might be relaxed, and amino acid changes could be allowed if 'social immunity' (Cremer et al., 2007) compensates this effect, positive selection driven repeated amino acid replacements at selected sites need to be detected more often if the high parasite pressure is especially severe in social insects (Viljakainen et al., 2009).
(3) Finally, some AMPs might attack their targets in such a way that evolving resistance is not possible without coordinated changes at many microbial genes. Selection on AMPs would primarily occur when hosts enter new niches and are forced to adapt to novel pathogen species not previously encountered (Tennessen, 2005).

### 4.3. Conclusion

Host and social parasite bumblebee immune system genes undergo more purifying selection, but also positive selection, than non-immune system genes. The combination of social defence mechanisms ('social immunity') and the evolution of physiological defence mechanisms form the current model of a combined social insect defence system against parasites and pathogens, which might compensate for the low number of immune genes and isoforms of AMPs. Additional (social) insect genomes, including comparative studies of immune system related gene evolution, will be needed to finally understand host–parasite mediated defence adaptations and selection occurring on host immune systems.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.02.002.

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