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Validation of genetic markers associated with chalkbrood resistance

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ABSTRACT

Chalkbrood is one of the major fungal diseases of honey bee brood. Systemic mycoses caused by the fungus *Ascosphaera apis* may significantly reduce brood population, and consequently, colony strength and productivity. Developing genetic marker(s) associated with the enhanced brood survival will be useful for breeding bees with resistance to this fungal disease. In this study we tested previously identified genetic markers (AMB-00612262, AMB-00858654) with the highest LOD (logarithm of odds) significance threshold value in a single mapping population of Russian honey bee stock that exhibits a variety of chalkbrood responses. Two additional single nucleotide polymorphisms (SNPs) (AMB-00858574, AMB-01151447) flanking this region were investigated to strengthen the potential correlation of the genotype to chalkbrood resistance (ChbR) phenotype. The objective of this study was to determine whether these biomarkers correlate with the ChbR phenotype in populations of the honey bee other than the original mapping population of Russian honey bees. Genetic markers showing the strongest association with the ability of honey bee larvae to survive fungal infection can be tested in Marker Assisted Selection (MAS). Our results suggest that there is no statistically significant correlation between the four genetic markers and chalkbrood survival in any of the honey bee stocks (Russian, VSH and Carniolan) tested in this study.

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KEYWORDS: honey bee, chalkbrood, *Ascosphaera apis*, QTL, genetic marker, RFLP

INTRODUCTION

The mechanisms of physiological resistance to infectious diseases are very complex and potentially multifactorial that may rely on the activation of innate immune and stress responses, and involve energy re-allocation and tissue-specific developmental adaptations. In that respect, mechanisms of resistance to pathogens are fundamentally different from resistance to pesticides where a single nucleotide change is known to confer resistance to a specific class of pesticides [1, 2].

A large variety of pathogens is known to infect honey bees, contributing to colony losses. Among them, brood diseases present a significant concern since they affect bees at the most vulnerable developmental stage and often lead to rapid depopulation of the colony. Traditional selection of honey bee stocks with increased resistance to infectious diseases via classical genetics has been used successfully [3-5]. However, this type of selection is based entirely on phenotype of the colony as a whole without consideration for susceptible life-stages or use of known genetic mechanisms of resistance and their corresponding molecular biomarkers as a means to assist in the selection process. More recent studies take advantage of the availability of the honey bee genome to identify quantitative trait loci (QTL) and to develop genetic markers associated with the disease resistance in honey bees [6-8].

The development of genetic tools for chalkbrood resistance (ChbR) in honey bees has been a primary focus of investigations by Holloway *et al.* [6, 7] and Aronstein and Holloway [9]. Holloway *et al.* [7] identified QTL (Amel_4.5 LG 11) associated with increased survival of honey bee larvae in a single mapping population of Russian honey bees infected with the chalkbrood fungus *Ascosphaera apis.* The subsequent fine mapping study [6] identified two single nucleotide polymorphism (SNP) markers (AMB-00612262; NCBI rs44679060 and AMB-00858654; NCBI rs44916427) associated with the variable resistance. Validation of these ChbR SNPs in the general population of Russian bees and other commonly used honey bee stocks will allow practical application of these genetic markers in a marker assisted selection (MAS) program. The main objective of this study, therefore, is to evaluate the ChbR SNPs to determine whether they correlate with resistance to chalkbrood in honey bee populations other than the original mapping population.

METHODS

Bee resources

Honey bee colonies were established in early spring of 2014 and maintained in USDA-ARS (US Department of Agriculture-Agricultural Research Service) Honey Bee Breeding, Genetics & Physiology Laboratory (Baton Rouge, LA) apiaries, all located within a 1 km radius. Three stocks (three colonies per stock) of bees were tested: Russian (selected for Varroa mite resistance in a closed population from honey bees sourced from Eastern Russia), honey bees with the Varroa Sensitive Hygiene (VSH) trait (outcrossed to *Apis mellifera ligustica*) and a commercial source of Carniolan (*A. m. carnica*) honey bees. Colonies were managed using routine beekeeping practices except that no in-hive chemicals were used to aid in honey bee survival. Colonies were examined for presence of diseases and parasites on a regular basis. In late fall, Carniolan bees had very high levels of Varroa mites and some clinical signs of deformed wing virus (DWV) that resulted in loss of most of the colonies by November 2014.

Fungal isolates and culture

A local *A. apis* strain (BBR) was isolated from archived infected larvae used in a chalkbrood QTL study [7] and purified in culture [10]. Identification was confirmed by polymerase chain reaction (PCR) using *A. apis* specific primers, AscoF3 (5'-GCACTCCCACCCTTGTCTA-3') and AapisR3 (5'-CCCACTAGAAGTAAATGATGGTTA-3') as described by [11]. Sequenced *A. apis* strains (ARSEF 7405 and 7406, ARSEF Culture Collection, Ithaca, NY) were used as controls in identification [12]. Isolates were grown on YGPSA (1% yeast extract, 1% glucose, 0.1 M KH_2PO_4, 1% soluble starch, 2% agar) solid culture medium [13] with 100 µg/ml ampicillin sodium salt and 6 µg/ml streptomycin sulfate antibiotics and incubated at 33 ºC for 7 days, and then at room temperature for 3 days. Ascospores were harvested from culture plates and stored at -20 ºC. A subset of spores was heat-treated at 70 ºC for 2 h. Viability of heat-treated spores was tested in culture by plating ~10^4 spores onto YGPSA and incubating as above to ensure no visible growth on culture plates. Heat-treated spores were stored at -20 ºC. Prior to larval inoculations, spores were tested for viability following the same protocol.

In vitro larval bioassay

In vitro studies can be used to separate behavioral resistance in social insects from physiological responses of individuals. All tests were conducted *in vitro* using the larval bioassay [10]. We collected 3-day-old larvae weighing between 18-38 mg from brood frames and placed them into 6-well cell culture plates lined with a circle of fine mesh fabric to keep larvae from sticking to the plate (5 larvae per well). The experiment contained three treatment groups: 1) larvae fed diet only, 2) larvae fed diet inoculated with heat-treated, non-viable *A. apis* spores (10^4 spores/larva), and 3) larvae fed diet inoculated with viable *A. apis* spores (10^4 spores/larva). Larvae were initially fed inoculum in larval diet [14] (150 µl/well). After 48 h post inoculation (hpi), larvae were moved to clean 6-well plates and fed pure diet as needed. Plates were incubated at 33 ºC and 97% relative humidity (RH) for the duration of the experiment.

Preliminary tests indicated that an initial dose of 10,000 viable spores/larva produced consistent infectivity with typical chalkbrood disease pathology [10] and larval mortality over 7 days, fitting a normal distribution in contrast to higher spore doses that lead to a skewed distribution of mortality with 100% mortality by 96 h (unpublished data).

The distribution of mortality we observed followed a bell-shaped curve with very few larvae dying within the first 48 h, disease onset and mortality beginning at 72 h, mortality numbers peaking between 96 h-120 h, followed by a trailing off in mortality numbers beyond 120 h.

Four replicate assays were conducted between May and September 2014. Each treatment consisted of 90 larvae from each stock, for a total of 270 larvae per treatment (N = 810 larvae total/assay).

Larval mortality test

Larval mortality, time to death and visible appearance of clinical signs of the disease were assessed for all treatment groups in the four independent assays. Mortality was recorded at 24 h intervals; dead larvae not showing clinical signs of the disease were incubated at 33 ºC on moistened blotting paper in petri dishes for 3-4 days to determine the cause of death. Fungal growth indicated that larvae consumed fungal spores and potentially died due to fungal exposure. Larvae showing fungal growth at the time of assessment were scored as susceptible. Larvae were rinsed in 1.5 ml microcentrifuge tubes containing 500 µl ice cold 0.9% saline solution, blotted on sterile paper towels, placed in new 1.5 ml tubes and stored at -20 ºC until further analysis. The experiment was terminated 24 h after more than 50% of the larvae pupated, 144-168 hpi. Remaining larvae and pupae from all treatment groups were scored as resistant and collected as above.

Validation of genetic markers associated with chalkbrood resistance

In this study we used RFLP (restriction fragment length polymorphism) analysis to validate genetic markers associated with chalkbrood resistance in a single mapping population of Russian bees [6] and to determine whether or not they may be correlated with resistance to chalkbrood in general populations of Russian, as well as, VSH and Carniolan stocks.

Two SNP markers (AMB-00612262/NCBI rs44679060 and AMB-00858654/NCBI rs44916427) were found in a major QTL (Amel_4.5 LG 11) associated with survival of honey bee brood in chalkbrood infected Russian bee colonies (Figure 1). Two additional SNPs (AMB-00858574 and AMB-01151447) flanking this region and found to be polymorphic in the original mapping population of Russian bees [6] were developed to strengthen the correlation of the genotype to ChbR phenotype (Figure 1).

Frozen larval tissue, < 50 mg, was placed in 1.5 ml microcentrifuge tubes with 300 µl solution of 1% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 7.5), 100 MM NaCl and 50 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.3-8). Tissue was crushed using disposable pestles (USA Scientific, Inc., Ocala, FL, USA) with ~45 mg of 0.1 mm Zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA). Samples were then incubated at 70 °C for 2 h followed by a standard phenol–chloroform DNA extraction method and ethanol precipitation. DNA pellets were washed with ice-cold 70% ethanol, dried and dissolved in 100 µl double deionized water (ddH_2O) each and stored at -20 °C for later analysis.

A DNA fragment of 300 bp from the AMB-00612262 SNP was PCR amplified using sequences flanking the SNP marker (Table 1). Amplification was performed in 20 µl final reaction volumes, containing 1.5 U of GoTaq Flexi DNA polymerase (Promega Co., Madison, WI, USA) with the green 5 × GoTaq Flexi buffer, 0.25 mM of the nucleotide triphosphates (dNTP) mix, 3.75 mM $MgCl_2$, 100 × bovine serum albumin (BSA), 0.25 µM of each primers, and 1 µl of gDNA. PCR conditions were as follows: after initial denaturing at 95 ºC for 3 min, 35 cycles of PCR were performed at 95 ºC (30 s) denaturing, 55 ºC annealing (30 s), extension at 72 ºC (45 s), and with a final extension of 72 °C (5 min) on MJ Research (Watertown, MA) model PTC200 thermal cycler. Enzymatic digest of PCR product with EcoRV (New England Biolabs, Inc., Ipswich, MA) followed. The digest products were visualized on 2% agarose gel. Similarly, AMB-00858654, AMB-00858574 and AMB-01151447 flanking DNA fragments were amplified using PCR and analyzed by RFLP using primers and restriction enzymes listed in Table 1.

The RFLP analyses of SNPs in this study were based on the presence or absence of specific restriction sites within the alternative alleles, see Table 1, SNP alleles. For example, in SNP marker AMB-00612262, homozygous resistant genotype RR produces a single 300 bp fragment; heterozygous genotype RS produces three bands of 300 bp, 213 bp and 87 bp; and homozygous susceptible genotype SS produces

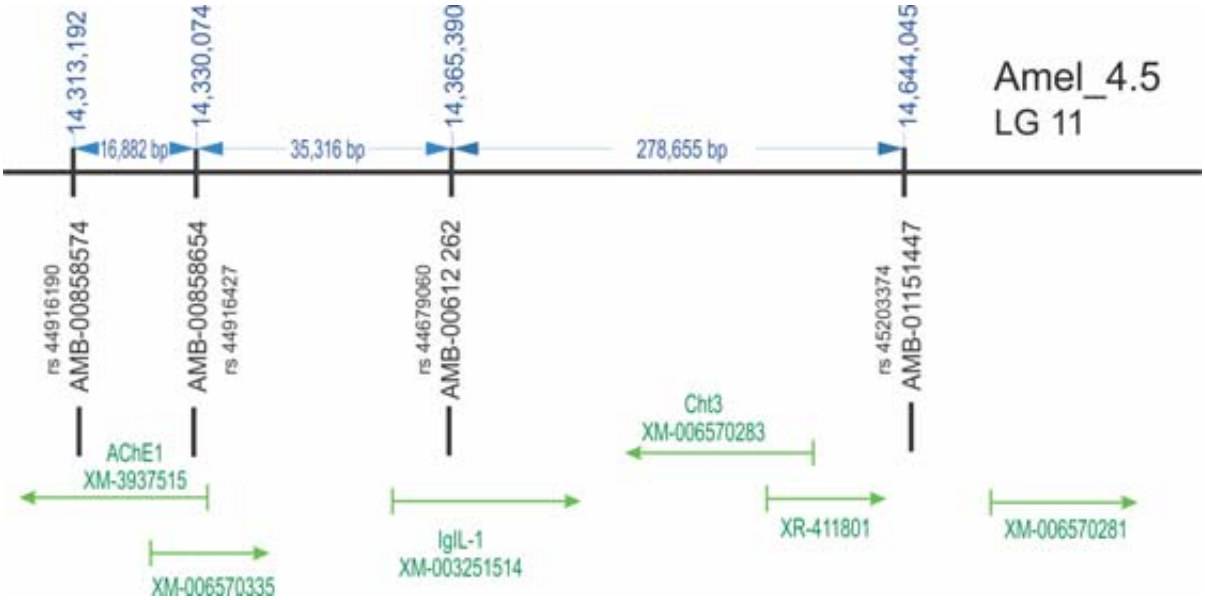

Figure 1. Amel_4.5 LG 11 map showing genomic location of SNPs associated with chalkbrood resistance (rs44679060 and rs44916427) [6] and two additional SNPs flanking this region (rs44916190 and rs45203374). Numbers (blue) above the line indicate location and distances between SNPs; NCBI accession numbers (black) are shown below the line. Arrows (green) below the line show location and transcription direction of the genes containing the SNPs, gene names (if known) and NCBI accession numbers.

Table 1. SNPs tested in this study for validation of the potential association with larval resistance to chalkbrood disease.

BMC-HGSC SNP ID/NCBI ID	PCR Primer sequences	Restriction enzyme	Digest fragment sizes	SNP alleles
AMB-00612262 /rs44679060	TGGCTACAAACTGTTGCTCCATCT GAGAGCGCGAAATCACCGATGAAA	EcoRV	300 (213/87)	C/T
AMB-00858654 /rs44916427	GGTCTCCAACATTTGTTCAGATTCGAC GCAACTCGAGATGATTTCGCGCAT	BstBI	307 (115/192)	C/G
AMB-00858574 /rs44916190	TCATTGTTCCCACCGATCGAGCAT CGCTGTTTGGCATTCGACACTTTC	BstBI	309 (115/194)	C/T
AMB-01151447 /rs45203374	ATGATTCGCCTTGAACTTGCGACC TGAACCTCAAAGACTACCACGCCA	NruI	368 (124/244)	C/T

two DNA fragments of 213 bp and 87 bp sizes (Figure 2, Table 1).

Statistical analysis

Phenotypic designations of susceptible and resistant larvae were generated using survival data from larval mortality tests (above). Resistant and susceptible pools of bee larvae were analyzed for correlation to the R(QTL) markers [6]. Statistical analyses were done using SAS/STAT® software 9.4 for Windows (SAS Institute Inc. Cary, NC, USA). All statistical significant differences inferred in this study were determined at the 5% confidence level. SAS PROC GLIMMIX was used to perform analysis of variance (ANOVA) with Kenward-Rogers adjustment of degrees of freedom and fitting a binomial distribution on survivorship of larvae at specific times, and to look at stock differences in larval mortality over time in susceptible individuals within the viable spore treatment group.

RESULT AND DISCUSSION

Validation of chalkbrood resistance markers

Analysis of genotypes (RR, RS, SS) associated with the SNP marker (AMB-00612262) by stocks within susceptible and resistant phenotypes showed that there was a significant difference in frequency of RR genotype by stock ($F = 9.25$; df = 2, 10.16; $P = 0.01$) with VSH having the highest frequency at approximately 85% and Russian having the lowest frequency at about 27% (Table 2). There was no significant difference in frequency of RR between phenotypes across stocks ($F = 0.12$; df = 1, 6.95; $P = 0.74$) or within stocks (Carniolan: $F = 1.17$; df = 1, 4.97; $P = 0.33$ Russian: $F = 0.90$; df = 1, 6.6; $P = 0.38$ VSH: $F = 0.21$; df = 1, 9.46; $P = 0.65$). The SS genotype occurred too infrequently in Carniolan and VSH to calculate a statistic. There was no significant difference in frequency of the SS genotype between Russian phenotypes with 31% of the larvae being resistant and 40% being susceptible (Std Err 15 and 9%, respectively). Those same patterns of genotypic frequencies within the stocks were present in all treatment groups and throughout all assays (Figure 3). We also found that genotypic frequencies among susceptible individuals did not change based on the time of death. On comparing genotypes from individuals collected at the final time point and 48 h prior, which corresponded to the time point of peak mortality, the data showed no significant differences due to a stock, time, or interaction effect ($F = 12.21$; df = 2, 1; $P = 0.20$; $F = 0.01$; df = 1, 1; $P = 0.95$; and $F = 0.64$; df = 2, 1; $P = 0.66$, respectively). The other three genetic markers were not polymorphic or informative in study populations.

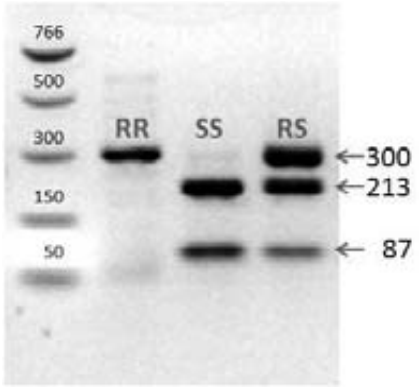

Figure 2. SNP marker (AMB-00612262) associated with chalkbrood resistance: Homozygous resistant genotype RR produces a single 300 bp fragment; heterozygous genotype RS produces three bands of 300 bp, 213 bp and 87 bp; and homozygous susceptible genotype SS produces two DNA fragments of 213 bp and 87 bp sizes.

The genetic markers AMB-00612262 and AMB-00858654 associated with chalkbrood resistance previously described by [6] showed no correlation

Table 2. Summary of results for PROC GLIMMIX for % RR genotype for AMB-00612262. LSMEANS (Mean % RR) with the same letter are not significantly different.

Stock	Phenotype	Mean % RR		Std Err %
Carniolan		57.35	B	8.00
Russian		27.21	C	7.64
VSH		85.34	A	4.82
Carniolan	Susceptible	55.06		9.96
Carniolan	Resistant	59.61		12.42
Russian	Susceptible	36.55		10.99
Russian	Resistant	19.53		9.57
VSH	Susceptible	87.67		5.83
VSH	Resistant	82.65		7.88
	Susceptible	63.13	a*	6.39
	Resistant	54.44	a	8.01

*$P = 0.4156$ (test of least significant difference).

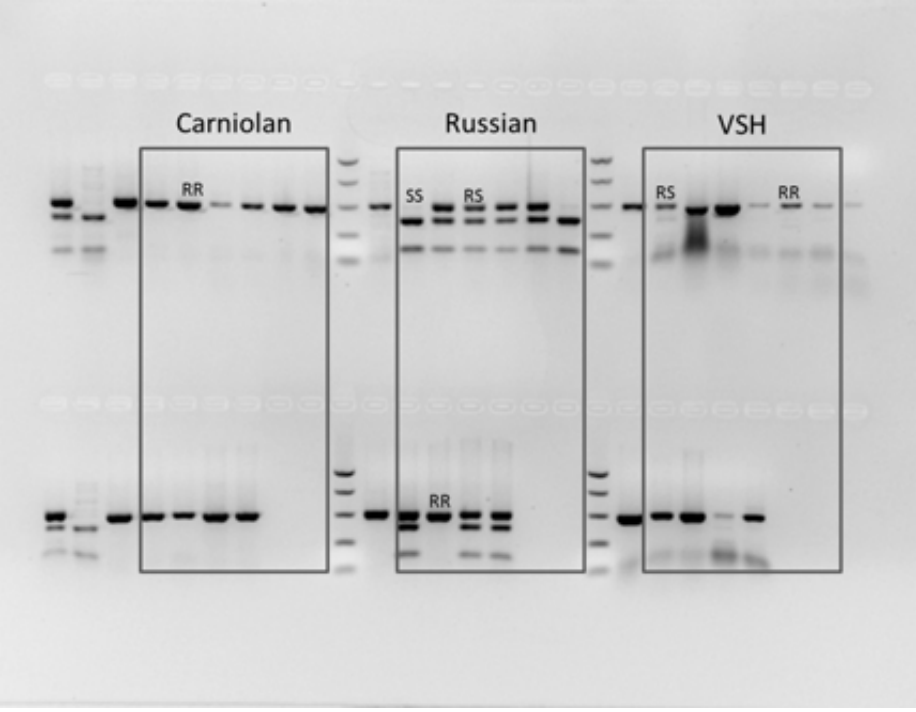

Figure 3. Gel electrophoresis of SNP marker (AMB-00612262) illustrates typical allele profiles observed within each stock. Genotypes by stocks within susceptible and resistant phenotypes showed a significant difference in frequency of RR allele by stock. While RR allele was not observed in the original mapping population of Russian bees, it was present in our population, but at a much lower frequency than in Carniolan and VSH bees. The SS allele occurred too infrequently in Carniolan and VSH to calculate a statistic.

to susceptible or resistant phenotypes in our study populations of Carniolan, Russian, and VSH honey bees. Therefore, there was no validation of the markers beyond the mapping population of Russian bees in which they were initially identified.

Genetic markers

Fine mapping of the QTL region that produced the highest LOD of 3.1 identified two SNPs (AMB-00612262 and AMB-00858654) that significantly associated with resistance to chalkbrood phenotype [6]. Here we evaluated the association of these SNPs with the larval phenotype in general and with non-mapping populations of three honey bee stocks, including Russian honey bees (used in the original mapping study [7]), Varroa sensitive hygiene trait (VSH) bees and Carniolan. Analyses of two additional SNPs also showed no correlation with larval ability to survive fungal infection.

In summary, none of the genetic markers tested in this study showed predictive capabilities of larval phenotype in any of the general honey bee stocks. Since SNPs did not provide significant correlations with larval survival or time to death in response to chalkbrood infection, we do not consider them to be useful for marker assisted selection when traditional selective breeding is not performed first.

There could be a number of explanations for this outcome. Only a single mapping population was used in the original study with the highest statistical significance LOD value of 3.15 [7]. As a consequence, too low LOD value threshold in QTL analysis may result in false-positive QTL, especially when no other population is available to verify the phenomenon. The initial mapping population was developed during a particularly devastating chalkbrood season and those honey bees that were resistant may have been carrying serendipitously

strong or unique resistance mechanisms that are not normally present in current general populations. Such a phenomenon would inhibit further study, especially in situations where many generations separate the study populations, or if the initial resistance mechanism was physiologically costly in some way and easily lost from the population. Since disease resistance is often thought to be regulated by multiple genes working in concert, analysis of additional segregating populations could increase the possibility of identifying other QTL or genes in the pathway, or independently acting QTLs.

It is worth noting that developing SNP markers for one honey bee stock may not be useful in other bee stocks. All four SNP markers tested in this study were polymorphic and informative for the mapping population of Russian bee stock; however, they were neither polymorphic nor informative for VSH and Carniolan bees. Therefore, stock-specific markers associated with a trait of interest may be required for a MAS program.

CONCLUSION

This study validated previously identified [6] and two flanking SNPs (Amel_4.5 LG 11) for correlation of genotype to ChbR phenotype in three honey bee stocks. None of the SNPs showed significant correlation with larval survival or time to death in response to chalkbrood infection, and therefore will not be useful for marker assisted selection.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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