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Brief Communication

Allelic Variation in a Single Genomic Region Alters the Microbiome of the Snail *Biomphalaria glabrata*

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Abstract

Freshwater snails are the intermediate hosts for numerous parasitic worms which can have negative consequences for human health and agriculture. Understanding the transmission of these diseases requires a more complete characterization of the immunobiology of snail hosts. This includes the characterization of its microbiome and genetic factors which may interact with this important commensal community. Allelic variation in the Guadeloupe resistance complex (GRC) genomic region of Guadeloupean *Biomphalaria glabrata* influences their susceptibility to schistosome infection and may have other roles in the snail immune response. In the present study, we examined whether a snail's GRC genotype has a role in shaping the bacterial diversity and composition present on or in whole snails. We show that the GRC haplotype, including the resistant genotype, has a significant effect on the diversity of bacterial species present in or on whole snails, including the relative abundances of *Gemmatimonas aurantiaca* and *Micavibrio aeruginosavorus*. These findings support the hypothesis that the GRC region is likely involved in pathways that can modify the microbial community of these snails and may have more immune roles in *B. glabrata* than originally believed. This is also one of few examples in which allelic variation at a particular locus has been shown to affect the microbiome in any species.

Subject areas: Gene action, regulation and transmission, Molecular adaptation and selection
Keywords: allelic variation, *Biomphalaria glabrata*, microbiome, schistosomiasis

Planorbid snails transmit numerous mammalian parasites including liver flukes (such as *Fasciola* and *Chlonorchis*), lungworms (*Angiostrongylus*), intestinal flukes (*Echinostoma*), and most importantly blood flukes (*Schistosoma*) (Loker 2010; Giannelli et al. 2016). By transmitting these parasites, these intermediate molluscan hosts are essential for perpetuating diseases which are severely detrimental to human health and livestock productivity (Pearce and MacDonald 2002; Sokolow et al. 2016). Understanding the biology of the intermediate hosts of these diseases may be important for understanding mechanisms behind their transmission or survival and could provide future tools for preventing the spread of helminth parasites.

Biomphalaria glabrata is a snail of particular interest because it is the intermediate host for the human parasite *Schistosoma mansoni* in the America (Pearce and MacDonald 2002; Reardon 2016). Allelic variation of a single genomic region, the Guadeloupe resistance complex (GRC), in Guadeloupean *B. glabrata* (BgGUA) has been shown to correlate with the resistance of BgGUA to Guadeloupean *S. mansoni* (SmGUA) (Tennessen et al. 2015). The GRC region contains proteins with putative structures that resemble recognition proteins. A single transmembrane GRC protein, Grctm6, can influence the number of parasites released from infected snails (Tennessen et al. 2015; Allan et al. 2017a, 2017c). Additionally, recent findings have shown that allelic variation in the GRC can modify recognition responses to some pathogen-associated molecular patterns (PAMPS) (Allan and Blouin 2018). BgGUA has 3 distinct GRC haplotypes which have been suggested to be under balancing selection (Tennessen et al. 2015) and which show variable expression across snail populations (Galinier et al. 2017). Given that schistosomes are an introduced species in the America, the GRC must have originally evolved to protect against native pathogens. Thus, it is likely that allelic variation in the GRC evolved, and is maintained, to recognize a variety of pathogens and could have an important immune role outside of schistosome defense.

The microbial community of *B. glabrata* and other closely related planorbid snails has been characterized via cultivation methods and bacterial 16S rRNA sequencing (Ducklow et al. 1979, 1981; Van Horn 2012; Silva et al. 2013). These studies have found a diverse array of commensal bacterial phyla in these snails, but no studies have specifically examined the effects of any genotype or condition on the microbial community of these snails. Although most studies examining the interaction between genetics and the microbiome have examined the effects of the presence/absence of a gene or the association of genes with physiological phenotype, naturally occurring allelic variation in a single gene has primarily been associated with changes in the microbial community in humans (Khachatryan et al. 2008; Frank et al. 2011; Spor et al. 2011). For example, allelic variation in genes involved in the control of inflammation and pathogen recognition have been strongly associated with changes in commensal microbial communities and subsequent disease processes (Khachatryan et al. 2008; Frank et al. 2011). Given that GRC-encoded proteins are potentially involved in immune recognition processes and are present in hemocytes, the cells that are likely to maintain homeostatic control of commensal microbes in molluscs, it is possible that some aspect of the microbial community could be influenced by variation in the GRC region (Loker 2010; Allan et al. 2017b). Therefore, we tested whether snails with different GRC genotypes differ in microbial diversity and abundance.

In the present study, we observed that allelic variation in the GRC region can have a significant effect on the bacterial diversity and community composition at low taxonomic levels. Analysis of individual taxa showed an effect of GRC genotype on the relative abundances of *Gemmatimonas aurantiaca* and *Micavibrio aeruginosavorus*. These results are the first indication that the GRC region could have a role in the abundance of specific bacterial taxa of BgGUA's microbiome and the first evidence that allelic variation of a single genetic locus can influence microbial diversity or composition in a mollusc.

Materials and Methods

Animals

BgGUA was collected in 2005 from the island of Guadeloupe and maintained under standard conditions as previously described (Theron et al. 2008, 2014; Tennessen et al. 2015). For this study, we used inbred lines that were generated from this outbred population via 1–3 generations of selfing. All inbred lines were derived independently from each other, each from a randomly chosen snail from the same outbred population of BgGUA. Lines had been isolated from each other for a minimum of 3 years before these experiments. Each line is homozygous for 1 of the 3 GRC alleles, which we named *R*, *S1*, and *S2*, as previously described (Tennessen et al. 2015). Here, we used 12 *RR* lines, 7 *S1S1* lines, and 11 *S2S2* lines. Outbred BgGUA were maintained in 2 separate tanks that had been isolated from each other for 1 year before these experiments. All snails used in this study were housed and fed identically and were size matched (6–8 mm).

Study Design, and Sample Collection and Processing

Firstly, we analyzed multiple inbred lines of the 3 homozygous genotypes (no crosses or parent–offspring combinations, see above) to examine the diversity and community composition of bacterial taxa. We also analyzed the relative abundance of individual operational taxonomic units (OTUs). Secondly, we conducted a corresponding analysis on the 6 possible genotypes in the outbred snail population.

For the inbred line experiments, a single snail from each inbred line (12 *RR*, 7 *S1S1*, and 11 *S2S2)* was individually housed, starved for 24 h (Van Horn 2012), and frozen for DNA extraction. This experiment was repeated on 2 separate occasions, 1 month apart. For the second, independent test, outbred snails were sampled haphazardly from 2 separate tanks (without a priori knowledge of their genotypes), starved for 24 h, and frozen for DNA extraction. DNA extraction was done using the DNeasy PowerSoil Kit (Qiagen) and performed according to manufactures' instructions on whole snails. Sampled snails were genotyped at the GRC locus as previously described (Tennessen et al. 2015), and all samples were sent for 16S rRNA amplification. The numbers of individuals from the 6 possible genotypes in our sample from tank 1 were 5 *S2S2*, 7 *S1S2*, 4 *S1R*, 14 *RR*, 19 *S2R*, and 0 *S1S1*; and from tank 2 were 15 *S2S2*, 3 *S1S2*, 6 *S1R*, 10 *RR*, 12 *S2R*, and 1 *S1S1*. The *S1* allele is rare in our 2 outbred populations, and only a single homozygous *S1S1* individual was identified in the sampled snails. Therefore, we did not include the *S1S1*genotype in subsequent analyses.

16S rRNA Amplification, Sequencing, and Data Processing

16S ribosomal RNA genes were PCR amplified by the Center for Genome Research & Biocomputing at Oregon State University (OSU CGRB), following the Illumina 16S metagenomics sequencing library preparation guide (https://support.illumina.com). For each of 2 sample sets (inbred and outbred snails), 16S rRNA amplicons (V3 and V4 regions) were sequenced on a lane of the Illumina MiSeq at OSU CGRB with paired-end sequencing and 250 bp reads. Forward and reverse reads were merged into contigs with PANDAseq (Masella et al. 2012). We used Kraken (Wood and Salzberg 2014) to assign contigs to taxonomic categories. We identified phylum as the taxonomic category nested immediately below "Bacteria." For each sample, we tallied the number of contigs assigned to each phylum. We also identified OTUs as the smallest taxonomic category that could be assigned at the species level or lower, and we tallied contigs in each OTU.

Statistical Analyses

Inbred lines were each sampled at 2-time points. We considered these to be 2 random samples of a variable microbiome, and thus, we

merged contigs from both time points into a single effective sample. Individual outbred snails were randomly sampled from the 2 tanks. Snail genotype, tank, and their interaction were considered in all analyses.

We used the "prentice.test" function in the R package "muStat" (Wittkowski and Song 2010) to perform Friedman rank-sum tests on Shannon's diversity index (H) (Shannon 1997) across genotypes. In the inbred experiment, we tested only for a difference of genotype, and in the outbred experiment, we added tank as a blocking variable. We used the Adonis function of the R Package "vegan" (Oksanen 2017) to estimate permutational multivariate analysis of variance using distance matrices (PERMANOVA) based on Bray–Curtis dissimilarity to examine taxa composition. We tested for a significant overall effect of genotype and for an effect of each genotype versus all of the others. For the outbred experiment, we included tank as an additional variable. Data were presented via nonmetric multidimensional scaling analysis (NMDS) of OTUs generated using R Package "vegan" (Oksanen 2017).

Only 2 of the 3 homozygous genotypes were present in more than 1 sample in both the inbred and outbred experiments (*S2S2* and *RR*). To identify particular taxa showing significant differences and to compare between experiments, we tested for differences between these 2 genotypes. We restricted our analyses to taxa represented by at least 100 contigs per experiment (inbred or outbred) across these 2 genotypes, to maximize statistical power and to focus on taxa with a non-negligible ecological presence. We then transformed counts into proportions by dividing by the total number of contigs from each sample. Again, we used the "prentice.test" function in the R package "muStat" (Wittkowski and Song 2010) to perform Friedman rank-sum tests on each taxon. In the inbred experiment, we tested only for a difference between S2S2 and RR genotypes, and thus, this analysis was equivalent to a Wilcoxon rank-sum test. In the outbred experiment, we added tank as a blocking variable. We identified all taxa showing $P < 0.05$ in at least one experiment, and we used Fisher's method to combine the *P*-values across both experiments. For Bonferroni corrected α, we divided 0.05 by the number of taxa observed in both experiments and with at least 100 contigs across S2S2 and RR genotypes in at least one experiment. A Bonferroni correction was used to ensure the most conservative approach was taken, thus reducing the likelihood that a false positive for the effects of genotype was discovered.

Results

Inbred Lines: Allelic Variation in the GRC Region Has a Significant Effect on the Overall Community Composition and the Relative Abundance of *M. aeruginosavorus*

In the inbred lines, we observed an average of 62 234 bacterial contigs per sample (median = 57 263). We identified 20 high abundance phyla (Supplementary Figure S1) and 1202 total OTUs in the inbred microbiome (Figure 1A). Shannon's diversity index for OTUs showed no significant difference ($P > 0.10$) among inbred lines, although there was no significant effect of genotype on OTU composition overall (PERMANOVA, $R^2 = 0.13$, $P = 0.1$) (Supplementary Table S1). *S1S1* had significantly different community composition from *S2S2* and *RR* (PERMANOVA, $R^2 = 0.13$, $P = 0.03$) (Supplementary Table S1, Figure 1A). We observed 223 OTUs with enough contigs for individual analysis. A single OTU, *M. aeruginosavorus* ARL-13, showed a significant effect, both overall (Friedman rank-sum test, $\chi^2 = 17.2$, degrees of freedom = 2, uncorrected $P = 1.8e\text{-}04$; $\alpha = 0.05/223 = 2.2e\text{-}04$) and for *RR* versus other genotypes (Wilcoxon rank-sum test, $W = 196$, uncorrected $P = 6.0e\text{-}05$). This OTU and 16 others showed marginal effects (uncorrected $P < 0.05$) between S2S2 and RR genotypes (Supplementary Table S2).

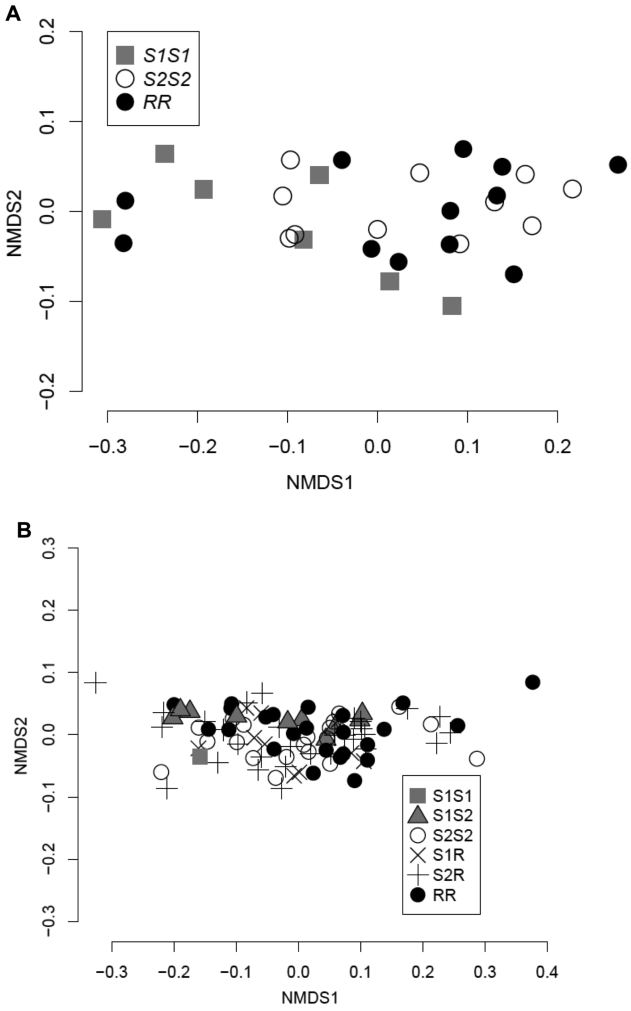

Figure 1. Allelic variation in the GRC region influences the microbiome of BgGUA. (**A**) OTU composition of the microbiome in homozygous inbred lines (isolated from each other for >3 years; *n* = 12 *RR*, 7 *S1S1*, and 11 *S2S2* lines) of BgGUA, as depicted with NMDS of OTUs generated. Overall community composition of S1S1 is significantly different (PERMANOVA Bray–Curtis, $P = 0.03$) from the other 2 genotypes, although all 3 genotypes show similarly high variability and did not have differing diversity (H). The relative abundance of *Micavibrio aeruginosavorus* ARL-13 is significantly different in *RR* lines (uncorrected $P = 1.8e\text{-}04$; $\alpha = 0.05/223 = 2.2e\text{-}04$). (**B**) OTU composition of the microbiome in outbred BgGUA (2 tanks separated for >1 year) (tank 1: *n* = 5 *S2S2*, 7 *S1S2*, 4 *S1R*, 14 *RR*, 19 *S2R*; tank 2: *n* = 15 *S2S2*, 3 *S1S2*, 6 *S1R*, 10 *RR*, 12 *S2R*), as depicted with NMDS. Allelic variation in the GRC region has no significant influence on the OTU composition or abundance but a significant difference in diversity (H, $P = 0.04$). All genotypes show similarly high variability.

Outbred Tanks: Allelic Variation in the GRC Region Has a Significant Effect on Bacterial Diversity

In the outbred samples, we observed an average of 53 684 bacterial contigs per sample (median = 45 117). We identified 20 high abundance phyla (Supplementary Figure S1) and 1231 OTUs in

the outbred microbiome (Figure 1B). Shannon's diversity index for OTUs showed a slight significant difference (P = 0.04) among genotypes (Supplementary Table S1). There was neither overall significant effect of genotype on OTU composition, assessed by Bray–Curtis dissimilarity, (R^2 = 0.05, PERMANOVA, P > 0.1), nor any significant effect of each individual genotype compared with the other 5 (PERMANOVA, P = 0.1) (Supplementary Table S1, Figure 1B). There was a significant environmental effect of tank (PERMANOVA, R^2 = 0.05, P < 0.05) and no significant effect of the interaction between genotype and tank (PERMANOVA, R^2 = 0.07, P = 0.08). We observed 223 OTUs with enough contigs for individual analysis. No OTU showed an individual significant effect, but 16 OTUs showed marginal effects (uncorrected P < 0.05) between S2S2 and RR genotypes (Supplementary Table S2).

Comparisons Between Inbred and Outbred Experiments for Particular OTUs

Out of 223 OTUs that we compared in S2S2 and RR snails across experiments, there were 17 (8%) showing marginal significance (P < 0.05, but not significant with corrected α) in the inbred lines and 16 (7%) showing P < 0.05 in the outbred snails (Supplementary Table S2, Figure 2). Assuming independence among OTUs, we would expect at most a single OTU to show marginal significance across both experiments by chance, but in fact, 4 OTUs did: *M. aeruginosavorus* ARL-13, *G. aurantiaca* T-27, *Parabacteroides distasonis* ATCC 8503, and *Halobacteriovorax marinus* SJ. Combining the P-value of both experiments with Fisher's method, only *M. aeruginosavorus* ARL-13 (P = 2.4e-05) and *G. aurantiaca* T-27 (P = 9.9e-05) were significant after correction (Bonferroni corrected α of 2.2e-04), although *P. distasonis* ATCC 8503 was very close to significance (P = 2.9e-04) (Figure 2). Oddly, though *M. aeruginosavorus* ARL-13 was the most significant, it did not show a consistent trend. In the inbred experiment, it was 1.7-fold more abundant in RR snails relative to S2S2 snails, while in the outbred experiment, it was 1.6-fold more abundant in S2S2 snails relative to RR snails. In contrast, both *G. aurantiaca* T-27 and *P. distasonis* ATCC 8503 showed consistent trends across experiments, with the largest effect being seen in *G. aurantiaca* T-27 (9-fold S2S2:RR enrichment in inbred snails, 8-fold S2S2:RR enrichment in outbred snails) (Supplementary Table S2, Figure 2). Thus, we show a significant effect of genotype on OTU composition, with the largest consistent trend occurring in *G. aurantiaca* T-27.

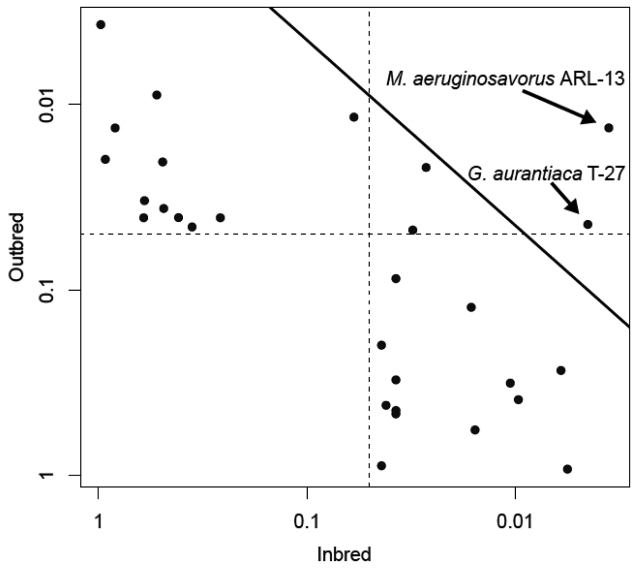

Figure 2. Allelic variation in the GRC region influences the relative abundance of *Micavibrio aeruginosavorus* and *Gemmatimonas aurantiaca*. *P*-values for the top 29 OTUs in inbred and outbred experiments (Wilcoxon rank-sum test). All OTUs with P<0.05 in either experiment are shown, plotted on a negative log scale for each experiment. The dotted lines represent P = 0.05. The solid line shows where combined *P*-values are significant at a Bonferroni corrected α of 2.2e-04. Two OTUs show a significant combined *P*-value (Fisher's combined *P*): *M. aeruginosavorus* ARL-13 (P = 2.4e-05) and *G. aurantiaca* T-27 (P = 9.9e-05).

Discussion

Though environmental, stochastic, and pathogenic factors have been repeatedly shown to have strong effects on the microbiome in multiple species and tissues, the study of the interaction between host genetics and the microbiome is still in its infancy (Spor et al. 2011; Thaiss et al. 2016). Understanding how the host genotype can modify the assemblage of the microbiome is essential for helping to characterize the immunobiology of any organism and could be particularly important in defining how those organisms respond to immune challenges (Spor et al. 2011; Gendrin et al. 2015; Kay et al. 2015; Mutapi 2015). BgGUA is an intermediate host of schistosomes in the America, and the GRC region appears to be important for its defense against these parasites. We aimed to determine whether this genomic region, which has been suggested to be involved in immune pathways beyond its defensive roles during schistosome challenge (Tennessen et al. 2015; Allan et al. 2017a), had any influence on the whole snail microbiome. Despite a relatively small sample size and the use of a very conservative statistical approach, our findings indicate that allelic variation in the GRC region has an effect on the diversity, community composition, and the abundances of at least 2 taxa in the whole snail microbiome of BgGUA. We found a significant effect of GRC genotype on the relative abundances of strains of *M. aeruginosavorus* and *G. aurantiaca* in 2 independent experiments. Thus, allelic variation at this locus can play a role in modifying the microbiome of BgGUA. These results also suggest that the genetic background of *B. glabrata* may have consequences for its commensal microbial community and specifically point to a role for genes in the GRC region in biotic interactions, including defense against pathogens.

There was a strong environmental effect (tank source) (Figure 1B), which is not surprising given that some of the most prominent factors affecting the microbiome of any species are environmentally driven (Spor et al. 2011; Rooks and Garrett 2016; Thaiss et al. 2016). Despite this environmental noise, our findings add to the small number of reports showing that variation at a single genetic locus can have significant effect on the microbiome of an organism (Khachatryan et al. 2008; Frank et al. 2011). One study found that the gut microbiome of humans can shift depending on their genotype of an intracellular pattern recognition receptor, *NOD2* (Caruso et al. 2014). The putative structure of some of the proteins encoded in the GRC region suggests that they may be transmembrane receptors (Tennessen et al. 2015; Allan et al. 2017a). Given that this locus is associated with schistosome resistance (Allan et al. 2017a) and galactose recognition (Allan and Blouin 2018), it is likely that these proteins may be involved in pathogen recognition, not dissimilar to the general immune role of *NOD2*. Therefore, we hypothesize that the genotypic effects of the GRC on *M. aeruginosavorus* and *G. aurantiaca* abundance, as well as the community composition and diversity of the

microbiome, may be the result of the role that the GRC plays in pathogen recognition.

The present study lacked sufficient power to definitively determine whether genotype affected the relative abundance of specific taxa in the outbred tanks. However, when we analyzed our inbred and outbred experiments together, we verified that the relative *M. aeruginosavorus* ARL-13 abundance was altered by GRC genotype and found additional effects for GRC genotype on *G. aurantiaca* T-27 relative abundance. *Gemmatimonadetes* is a recently discovered, Gram-negative phylum of bacteria which contains very few described members despite its early divergence from other bacteria (Zhang et al. 2003; Takaichi et al. 2010; DeBruyn et al. 2013). Though our findings show that *G. aurantiaca* T-27 abundance is positively associated with the *S2* allele, they also suggest that other taxa have a role in driving the overall association between GRC genotype and microbial composition. We found that *M. aeruginosavorus* ARL-13, a predatory Gram-negative bacteria known for disrupting other bacterial populations (Wang et al. 2011), was the only species found to be significantly different in both our inbred and our combined analyses. As we expected to find the similar taxa to vary according to genotype in both the inbred and outbred populations, these findings provide strong support that specific taxa have modified relative abundances due to GRC genotype alone. Interestingly, *RR* inbred snails had a greater relative abundance of *M. aeruginosavorus* ARL-13, relative to *S2S2*, in the inbred group but the inverse in the outbred snails. This inconsistent directional trend suggests the overarching effects of environment can skew the abundance of some of these taxa but that there are still underlying genotypic effects. It should also be noted that strain and species designations can be inaccurate when identified using short 16S rRNA. Therefore, it is important to compare the species/strain-specific differences in relative abundances in this study with those done in the future before hard conclusions regarding specific differential abundances should be made.

Finally, *RR* snails were found to be different from all *SS* snails in our inbred experiment, which could indicate that snails resistant to schistosome infection may have specific difference relative to those that are susceptible to schistosomes, and could indicate possible overlap between schistosome resistance and microbiome composition. Determining how these specific groups of bacteria interact with the GRC region, each other, other members of the microbial community, and other infectious pathogens will require more extensive microbiome characterization using much larger snail populations. Work extending from this study should also examine the potential effects of allelic variation on tissue-specific microbiomes (shell, integument, gut, etc.) as subtle changes in these tissues may be missed by whole snail analyses.

This study expands our basic knowledge of the microbiome of snails which are an intermediate host for human pathogens and is one of a handful of recent studies that show an effect of allelic variation within a population on host microbiomes. It also adds more data suggesting that genes in the GRC region code for proteins have important roles in biotic interactions, including host defense. The idea that these genes could influence pathogens indirectly, via effects on the microbiome, is another intriguing possibility that deserves consideration.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

We have made the primary data (read counts) used in these analyses available in Supplementary Tables S3 and S4.

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