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Alleles 177 (AC117), 263 (Ap243), and 269 (SV185) Confer Reduced Nosema Infection in Apis mellifera mellifera: Implications for Marker-Assisted Selection

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ABSTRACT

Microsporidian parasites of the genus Nosema, especially Nosema ceranae, continue to represent major threats to the health of Apis mellifera colonies. A promising strategy to mitigate these effects involves breeding honey bee colonies resistant to Nosema infection through molecular breeding methods such as marker-assisted selection (MAS). To enable this, it is necessary to establish reliable genetic markers for bee selection. This study aimed to investigate correlations between certain microsatellite loci and susceptibility to Nosema infection in the dark forest bee, Apis mellifera mellifera. Among the tested loci, AC117, Ap243, and SV185 were the most promising molecular markers associated with resistance to nosemosis. Their alleles "177," "263," and "269," respectively, correlated with a lower infection level. This work represents the first associative investigation aimed at identifying DNA loci linked to resistance against nosemosis in dark forest bees. The discovered microsatellite markers may serve as predictive tools for estimating the likelihood of Nosema disease occurrence.

Keywords: Nosema disease, Dark forest bee, Apis mellifera mellifera, microsatellite loci, Genetic association

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Introduction

Over recent decades, global honey bee populations have experienced adverse trends such as extensive colony losses and hybridization. These losses, referred to as colony collapse disorder (CCD), result from diminished adaptability of bees to environmental conditions and pose a serious concern to apiculture worldwide [1, 2]. CCD is thought to be multifactorial, influenced by pathogens such as Nosema, pollution, pesticide exposure, climatic factors, and various agricultural and beekeeping practices [3-5]. To prevent large-scale population declines caused by parasites and infections, maintaining genetic diversity in bee populations is crucial [6, 7].

At the same time, molecular approaches—like marker-assisted selection (MAS)—allow breeders to identify colonies with desirable traits (e.g., resistance to pathogens, docility, high productivity) or to eliminate undesirable ones (e.g., aggression, swarming) [8–11]. However, MAS remains a novel technique in apiculture, and no specific genetic markers have yet been widely accepted for practical breeding [12, 13].

The identification of DNA loci or genes related to economically valuable and adaptive traits (association mapping) is of high relevance. The most effective method involves genotyping large sets of molecular markers to discover genomic regions linked to traits of interest and to detect causal genes [14]. Once identified, such markers can be immediately employed in selective breeding to enhance disease resistance [15].

To date, quantitative trait loci (QTLs) associated with queen fertility [16, 17], resistance to chalkbrood [18, 19] and varroosis [20, 21], and different behavioral characteristics [22–24] have been identified. For instance, hygienic behavior—a social mechanism of disease control—plays a crucial role in reducing brood diseases like varroosis [25–27]. This disease, caused by the ectoparasitic mite Varroa destructor, remains one of the most harmful to honey bee brood [28–30]. Hygienic behavior confers considerable resistance to Varroa infestation [13, 30]. In total, more than 20 potential QTLs related to Varroa resistance behaviors have been identified across various genomic regions in honey bees [14, 20–22, 31].

Similar to varroosis, nosemosis represents another major honey bee disease [32–34]. However, studies exploring relationships between molecular genetic markers and nosemosis resistance are rare [35, 36].

Nosemosis is caused by microsporidia, obligate intracellular eukaryotic parasites that infect adult honey bees [37]. Nosema species proliferate within host cell cytoplasm, leading to extensive damage or complete destruction of the midgut epithelium [38, 39].

Two species of Nosema—N. apis and N. ceranae—are known to infect European honey bees. N. apis Zander, 1909 [40], an ancient parasite of A. mellifera, causes type A nosemosis, which is relatively mild and often manageable under favorable conditions [41–43]. N. ceranae Fries *et al*., 1996 [44], the agent of type C nosemosis, is a comparatively recent invader of A. mellifera [38, 44–46]. Initially detected in Apis cerana in Asia during the late 20th century [44], N. ceranae has spread globally among A. mellifera populations since 2006 [38, 41–43, 45–54]. Compared to N. apis, N. ceranae exhibits higher virulence and has been linked to significant colony losses, particularly in Mediterranean regions [32, 55–58].

Using microsatellite markers, four QTLs linked to reduced spore loads were identified in a Danish strain of Nosema-resistant bees [35]. These Buckfast colonies, selectively bred for decades to eliminate Nosema, demonstrate notable tolerance to infection [59, 60]. Unlike pure-line breeding, the Buckfast method combines different bee stocks to create hybrid colonies expressing favorable characteristics. Genetic contributions to Buckfast bees primarily originate from A. m. ligustica and A. m. mellifera, with other subspecies also incorporated. Consequently, expression of traits can vary significantly within this hybrid population [61].

It is recognized that bee subspecies, lines, and colonies differ in disease resistance, largely due to social immunity mechanisms such as grooming and hygienic behaviors [13, 15, 30, 62–70]. For instance, A. mellifera populations of Africanized origin show higher levels of these behaviors than European bees, which likely contributes to their stronger resistance to V. destructor [71].

Although some studies report no direct impact of N. ceranae on hygienic behavior [72], it is evident that the natural resistance of honey bees to Nosema is influenced by multiple factors, including genetic variation. The present research therefore aimed to identify associations between microsatellite locus variants and susceptibility or resistance to Nosema infection in dark forest bees (Apis mellifera mellifera).

Materials and Methods

Bee samples

This work examined *Apis mellifera mellifera* (dark forest bee) specimens collected from several Siberian locations situated between 81°29′–92°08′ E longitude and 50°44′–65°47′ N latitude. This subspecies, native to Europe, was first introduced to Siberia roughly 230 years ago and has since adapted successfully to the region's climatic and botanical conditions. The Siberian population is considered a managed group, as winter survival is ensured through human intervention [73].

Worker bees were sampled from 12 apiaries between late May and August 2016. In total, 226 individuals representing 28 colonies (8–10 bees per colony) were evaluated.

For *Nosema* detection, forager bees were specifically selected at hive entrances, since this group exhibits the highest probability and intensity of infection [39]. All samples were preserved at −20 °C pending analysis.

Study design

The experimental framework was divided into three main stages.

During the first phase, the presence of *Nosema* spp. was screened by both microscopic examination and polymerase chain reaction (PCR).

At the second phase, differences in genetic variation among bees showing varying degrees of *Nosema* infection were analyzed through polymorphic microsatellite loci. Prior studies on Siberian dark forest bees had

characterized their genetic diversity and identified suitable microsatellite markers [74]. For this investigation, 23 polymorphic loci were selected to detect allelic variants possibly linked with resistance to *Nosema* infection.
The final phase assessed the relationship between the identified microsatellite variants and nosemosis infection intensity using the odds ratio (OR) analytical method.

Experimental procedures
Each bee's midgut was removed and halved. One half was crushed in 0.5 mL of sterile distilled water, and *Nosema* spores were counted under a Zeiss Axio Lab.1 light microscope.
The remaining half was used for DNA extraction with the PureLink™ Mini kit (Invitrogen, Carlsbad, CA, USA) following the provided instructions. PCR reactions were performed on a MyCycler T100 thermal cycler (Bio-Rad, Foster City, CA, USA).
Detection of *Nosema apis* and *Nosema ceranae* was performed by duplex-PCR [42]. For *N. apis*, primers 321APIS-FOR (5′-GGGGGCATGTCTTTGACGTACTATGTA-3′) and 321APIS-REV (5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′) were used to amplify a 321 bp 16S rRNA fragment. For *N. ceranae*, a 218 bp fragment was amplified using primers 218MITOC-FOR (5′-CGGCGACGATGTGATATGAAAATATTAA-3′) and 218MITOC-REV (5′-CCCGGTCATTCTCAAACAAAAAACCG-3′) [42].
Each 20 µL PCR mixture contained 5–10 ng of template DNA, 1× buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, and 1 U Taq polymerase (Fermentas, Thermo Fisher Scientific, Chelmsford, MA, USA). Cycling conditions included: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min.
PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and visualized using a Gel Doc XR+ imaging system (Bio-Rad, Foster City, CA, USA). Positive controls (*N. apis* and *N. ceranae* DNA) and a negative control (ddH_2O) were included in every PCR batch.
Variation in 23 microsatellite loci — Ap066, K0457B, K1168, A007, A008, A028, A043, Ap049, Ap007, AC117, 6339, Ap068, Ap243, SV220, SV167, SV185, Ap226, H110, A024, AT139, A056, Ap249, and A113 — was assessed. Selection of loci was based on their polymorphism, broad chromosomal coverage (13 of 16 chromosomes), and literature linking them with disease resistance.
Fluorescently labeled primers were used as described by Solignac *et al*. [75]. PCR was performed in 20 µL reactions containing 5–10 ng DNA, 0.4 µM primers, 60 µM dNTPs, 1–2.5 mM $MgCl_2$, 1× buffer, and 1 U Taq polymerase (Fermentas, Thermo Fisher Scientific, Chelmsford, MA, USA). The thermal program comprised 3 min at 94 °C; 35 cycles of 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s; followed by 10 min at 72 °C [75].
PCR products were analyzed using an ABI Prism 3730 Genetic Analyzer and GeneMapper Software (Applied Biosystems, Foster City, CA, USA) at the Medical Genomics Center, Research Institute of Medical Genetics, Tomsk National Research Medical Center, Russian Academy of Sciences (Moscow, Russia). Two microliters of each PCR product were combined with GeneScan500-ROX size standard (Applied Biosystems, Inc.) and deionized formamide, then processed according to manufacturer protocols.

Evaluation of nosema infection levels
PCR diagnostics revealed that most bees were simultaneously infected by *Nosema apis* and *Nosema ceranae*. Therefore, total *Nosema* infection was considered collectively, without separating species. Spore counts were determined microscopically at 400× magnification in homogenized gut suspensions.
Bees were classified into infection categories: *Nosema*-negative (uninfected) and *Nosema*-positive (infected). Because precise quantification was unnecessary for the study's objectives, an approximate infection scale was applied. The *Nosema*-positive category was split into two intensity levels:

- Low infection: fewer than 100 spores visible per microscopic field.
- High infection**:** over 500 spores visible per field.

No intermediate (100–500 spores) group was included. As a result, three final categories were used for analysis: uninfected (*Nosema*-negative), mildly infected (*Nosema*-positive low), and heavily infected (*Nosema*-positive high).

Statistical evaluation

Each bee's genotype was used to estimate population-level parameters. Allelic and genotypic frequencies were tested for Hardy–Weinberg equilibrium (HWE), and the number of alleles per locus, observed heterozygosity (Ho), and expected heterozygosity (He) were computed in GENEPOP v.4.1 [76].

Within each infection category, Ho and He were compared using Student's *t*-test to evaluate differences in genetic variability. Allele and genotype frequencies were contrasted among groups with varying infection degrees using the chi-square test. When sample sizes were small, Yates' continuity correction was applied.

The relationship between allelic variation in microsatellite loci and *Nosema* infection was analyzed through the odds ratio (OR**)** and 95% confidence interval (CI) with the corresponding *p*-value [77]. Statistical significance was accepted at $p < 0.05$. OR values above 1 suggested a genetic variant increased disease likelihood, while OR values below 1 indicated possible resistance or protective effects.

Results and Discussion

Microsatellite-based Genetic variability in siberian A. m. mellifera

Bees showing different levels of *Nosema* infection were genotyped across 23 microsatellite markers. Comparison of allele distributions among the three infection groups (*Nosema*-negative, low, and high) revealed seven loci—AC117, A113, Ap243, A024, A007, Ap049, and SV185—as most relevant for subsequent analyses. Key genetic diversity indices, including allele and genotype frequencies, and both heterozygosity measures, are provided in **Table 1**.

Table 1. Allele/genotype frequencies and heterozygosity at seven microsatellite loci in dark forest bees according to *Nosema* infection status.

Locus	Genotype	Allele	Nosema-Negative Bees		Nosema-Positive Low		Nosema-Positive High	
			Genotype	Allele	Genotype	Allele	Gen	Allele
AC117	173–173	173	0.037	0.074 ± 0.036	0.024	0.079 ± 0.017	—	0.057 ± 0.021
	173–181	177	0.074	0.148 ± 0.048	0.110	0.075 ± 0.017	0.115	0.025 ± 0.014
	177–177	181	0.037	0.296 ± 0.062	0.008	0.260 ± 0.028	—	0.533 ± 0.045
	177–181	185	—	0.482 ± 0.068	0.039	0.587 ± 0.031	—	0.385 ± 0.044
	177–185		0.222	—	0.094	—	0.049	—
	181–181		0.259	—	0.142	—	0.475	—
	181–185		—	—	0.087	—	—	—
	185–185		0.370	—	0.496	—	0.361	—
	Ho/He		**0.296 ± 0.088 / 0.653 ± 0.040		**0.331 ± 0.042 / 0.577 ± 0.025		**0.164 ± 0.047 / 0.564 ± 0.025	
	N		27		127		61	
A113	210–218	210	—	—	0.016	0.008 ± 0.006	—	—
	212–212	212	—	0.107 ± 0.041	0.063	0.152 ± 0.023	0.016	0.063 ± 0.021
	212–214	214	0.036	0.018 ± 0.018	0.008	0.008 ± 0.006	—	—
	212–218	218	0.143	0.518 ± 0.067	0.125	0.598 ± 0.031	0.094	0.719 ± 0.040
	212–220	220	0.036	0.339 ± 0.063	0.023	0.207 ± 0.025	—	0.219 ± 0.037
	212–222	222	—	—	0.008	0.008 ± 0.006	—	—
	212–226	226	—	—	0.016	0.020 ± 0.009	—	—
	214–226	228	—	0.018 ± 0.018	0.008	—	—	—
	218–218		0.286	—	0.438	—	0.609	—
	218–220		0.321	—	0.172	—	0.125	—
	218–222		—	—	0.008	—	—	—
	220–220		0.143	—	0.109	—	0.156	—

	220–228		0.036	—	—	—	—	—
	226–226		—	—	0.008	—	—	—
	Ho/He		0.571 ± 0.094 / 0.605 ± 0.041		**0.383 ± 0.043 / 0.577 ± 0.027		0.219 ± 0.052 * / 0.432 ± 0.043	
	N		28		128		64	
Ap243	253–260	253	0.043	0.022 ± 0.022	—	—	0.074	0.037 ± 0.026
	256–256	256	0.305	0.413 ± 0.073	0.284	0.419 ± 0.035	0.519	0.593 ± 0.067
	256–263	260	0.218	0.109 ± 0.046	0.233	0.111 ± 0.022	0.074	0.167 ± 0.051
	256–266	263	—	0.239 ± 0.063	—	0.283 ± 0.032	0.074	0.056 ± 0.031
	256–269	266	—	0.022 ± 0.022	0.030	0.010 ± 0.007	—	0.037 ± 0.026
	256–272	269	—	0.109 ± 0.046	0.010	0.096 ± 0.021	—	0.037 ± 0.026
	260–260	272	0.043	0.022 ± 0.022	0.081	0.035 ± 0.013	0.111	0.056 ± 0.031
	260–263	275	0.043	0.065 ± 0.036	0.010	0.046 ± 0.015	—	0.019 ± 0.018
	260–266		0.043	—	0.020	—	—	—
	260–269		—	—	0.030	—	0.037	—
	263–263		0.043	—	0.132	—	—	—
	263–269		0.043	—	0.030	—	—	—
	263–272		0.087	—	0.010	—	0.037	—
	263–275		—	—	0.020	—	—	—
	269–269		0.043	—	0.020	—	—	—
	269–272		—	—	0.020	—	0.037	—
	269–275		0.087	—	0.040	—	—	—
	272–275		—	—	0.030	—	0.037	—
	Ho/He		0.565 ± 0.103 / 0.743 ± 0.043		**0.485 ± 0.050 / 0.719 ± 0.020		0.370 ± 0.093 * / 0.610 ± 0.067	
	N		23		99		27	
A024	92–92	92	0.500	0.712 ± 0.063	0.446	0.654 ± 0.030	0.313	0.578 ± 0.044
	92–100	96	0.231	—	0.177	0.008 ± 0.005	0.219	—
	92–106	100	0.192	0.154 ± 0.050	0.238	0.181 ± 0.024	0.313	0.266 ± 0.039
	96–96	102	—	—	0.008	0.008 ± 0.005	—	—
	100–100	106	0.038	0.135 ± 0.047	0.077	0.150 ± 0.022	0.156	0.156 ± 0.032
	100–102		—	—	0.015	—	—	—
	100–106		—	—	0.015	—	—	—
	106–106		0.038	—	0.023	—	—	—
	Ho/He		0.423 ± 0.097 / 0.452 ± 0.070		0.446 ± 0.044 / 0.517 ± 0.029		0.531 ± 0.062 / 0.571 ± 0.032	
	N		26		130		64	
A007	104–108	104	0.185	0.093 ± 0.039	0.224	0.121 ± 0.021	0.364	0.182 ± 0.041
	104–113	108	—	0.815 ± 0.053	0.017	0.797 ± 0.026	—	0.818 ± 0.041
	108–108	113	0.704	0.093 ± 0.039	0.655	0.082 ± 0.018	0.636	—
	108–113		0.037	—	0.060	—	—	—
	113–113		0.074	—	0.043	—	—	—
	Ho/He		0.222 ± 0.080 / 0.319 ± 0.075		0.302 ± 0.043 / 0.343 ± 0.036		0.364 ± 0.073 / 0.298 ± 0.052	

	N		27		116		44	
Ap049	120–120	120	0.036	0.161 ± 0.049	0.017	0.121 ± 0.021	—	0.057 ± 0.022
	120–127	127	0.250	0.714 ± 0.060	0.200	0.646 ± 0.031	0.113	0.745 ± 0.042
	120–130	130	—	0.054 ± 0.030	0.008	0.175 ± 0.025	—	0.085 ± 0.027
	127–127	139	0.536	0.071 ± 0.034	0.425	0.046 ± 0.014	0.585	0.085 ± 0.027
	127–130	152	0.036	—	0.192	0.013 ± 0.007	0.094	0.028 ± 0.016
	127–139		0.071	—	0.050	—	0.113	—
	130–130		0.036	—	0.067	—	0.019	—
	130–139		—	—	—	—	0.019	—
	130–152		—	—	0.017	—	0.019	—
	139–139		0.036	—	0.017	—	0.019	—
	139–152		—	—	0.008	—	—	—
	152–152		—	—	—	—	0.019	—
	Ho/He		0.357 ± 0.091 / 0.456 ± 0.071		0.475 ± 0.046 / 0.535 ± 0.032		0.359 ± 0.066 / 0.426 ± 0.057	
	N		28		120		53	
SV185	253–253	253	—	—	0.009	0.022 ± 0.010	—	—
	253–272	263	—	0.241 ± 0.058	0.027	0.313 ± 0.031	—	0.385 ± 0.050
	263–263	266	0.037	0.093 ± 0.039	0.134	0.094 ± 0.020	0.146	0.146 ± 0.036
	263–266	269	—	0.667 ± 0.064	0.045	0.549 ± 0.033	0.125	0.469 ± 0.051
	263–269	272	0.407	—	0.304	0.023 ± 0.010	0.354	—
	263–272		—	—	0.009	—	—	—
	266–266		0.074	—	0.045	—	—	—
	266–269		0.037	—	0.054	—	0.167	—
	269–269		0.444	—	0.366	—	0.208	—
	269–272		—	—	0.009	—	—	—
	Ho/He		0.444 ± 0.096 / 0.489 ± 0.061		0.446 ± 0.047 * / 0.591 ± 0.023		0.646 ± 0.069 / 0.611 ± 0.023	
	N		27		112		48	

N—number of bees analyzed per infection level; Ho—observed heterozygosity; He—expected heterozygosity under Hardy–Weinberg equilibrium. The table includes standard error estimates. Statistically significant differences between Ho and He are marked with (*) (*p < 0.05; **p < 0.001).

All examined loci were polymorphic. The fewest alleles occurred at A007 (three alleles), while A113 and Ap243 had the most (eight alleles each), giving an average of five alleles per locus **(Table 1)**.

Variation differed among infection groups for several loci. For instance, the main allele “256” at Ap243 varied significantly between the two infected groups ($t = 2.30$; $p < 0.05$). Another allele, “263”, from the same locus, differed between uninfected and highly infected bees ($t = 2.61$; $p < 0.05$), and also between low- and high-infected groups ($t = 5.10$; $p < 0.001$).

For AC117**,** alleles “177” and “181” showed significant frequency shifts between *Nosema*-negative and high-infection bees ($t = 2.46$; $p < 0.05$; $t = 3.09$; $p < 0.05$) and also between the two infected categories ($t = 2.27$; $p < 0.05$; $t = 5.15$; $p < 0.01$).

In nearly all loci, observed heterozygosity (Ho) was lower than expected (He), except for A007 and SV185 among heavily infected bees **(Table 1)**. Significant differences between Ho and He were found for A113 ($t = 3.82$, $p <$

0.001; $t = 3.16$, $p < 0.05$), Ap243 ($t = 4.35$, $p < 0.001$; $t = 2.09$, $p < 0.05$), SV185 ($t = 2.77$, $p < 0.05$), and AC117 ($t > 3.69$, $p < 0.001$).

Overall, screening of 23 microsatellite loci in the Siberian population of *A. m. mellifera* revealed specific markers—particularly AC117, A113, Ap243, and SV185—that appear promising for identifying genetic associations related to *Nosema* susceptibility or tolerance.

Comparative analysis of genetic diversity in A. m. mellifera under different infection levels

To assess the variability among bee groups differing in the degree of Nosema infection, comparisons of allele frequency distributions across microsatellite loci were carried out. Statistically significant distinctions were identified between infection categories.

For several loci—AC117, A113, Ap243, and Ap049—notable variations in total allele frequency distributions were detected among infection groups. Most of these differences appeared between Nosema-infected colonies (low versus high infection levels):

AC117: $\chi^2 = 44.61$, df = 7, $p < 0.01$

A113: $\chi^2 = 12.76$, df = 5, $p < 0.05$

Ap243: $\chi^2 = 19.77$, df = 7, $p < 0.01$

Ap049: $\chi^2 = 14.70$, df = 7, $p < 0.05$

Additionally, significant differences at AC117 were noted between uninfected and highly infected bees ($\chi^2 = 19.84$, df = 7, $p < 0.01$), while no meaningful differences were seen between uninfected and low-infected groups. At AC117, the alleles “177,” “181,” and “185” made the greatest contributions to the observed differences. The “177” variant was considerably more common among uninfected individuals than in highly infected ones ($\chi^2 = 9.59$, df = 1, $p < 0.01$). Conversely, “181” appeared more frequently in the heavily infected bees than in either the uninfected ($\chi^2 = 8.66$, df = 1, $p < 0.01$) or mildly infected groups ($\chi^2 = 26.56$, df = 1, $p < 0.01$). Differences in the frequency of “185” were recorded between the two infected categories ($\chi^2 = 14.17$, df = 1, $p < 0.01$).

For A113, allele “218” distinguished uninfected from severely infected bees ($\chi^2 = 6.81$, df = 1, $p < 0.01$). Further, between the two infected groups, significant frequency differences were detected for both “218” ($\chi^2 = 6.12$, df = 1, $p < 0.05$) and “212” ($\chi^2 = 6.60$, df = 1, $p < 0.01$).

In Ap243, the alleles “256” and “263” varied significantly across infection categories ($\chi^2 = 5.80$, df = 1, $p < 0.05$; $\chi^2 = 12.45$, df = 1, $p < 0.01$, respectively). The “256” variant dominated in the highly infected group, whereas “263” was characteristic of bees with lower infection levels. Moreover, “263” also differentiated the uninfected and highly infected groups ($\chi^2 = 6.98$, df = 1, $p < 0.01$).

At Ap049, alleles “127” and “130” contributed to the divergence between low- and high-infected bees ($\chi^2 = 4.00$, df = 1, $p < 0.05$; $\chi^2 = 5.12$, df = 1, $p < 0.05$). The “120” allele, meanwhile, distinguished uninfected bees from those with high infection intensity ($\chi^2 = 4.69$, df = 1, $p < 0.05$).

Although the total allele distribution for A007, A024, and SV185 showed no overall significant differences among infection groups, specific alleles did vary. Notably, the “269” allele of SV185 differed significantly between uninfected and heavily infected bees ($\chi^2 = 5.65$, df = 1, $p < 0.05$).

Association between genetic variants and nosema infection in dark forest bees

To identify alleles potentially linked with Nosema susceptibility or resistance in A. m. mellifera, odds ratios (OR) were computed **(Table 2)**.

Table 2. Comparative evaluation of allele frequencies associated with possible resistance or susceptibility to nosemosis in dark forest bees (A. m. mellifera).

Locus	Compared Alleles/Genotypes	Parameter	Nosema-Negative vs. Low-Positive	Nosema-Negative vs. High-Positive	Low-Positive vs. High-Positive
AC117	Allele 177 vs. others	OR	0.46	0.16	0.35
		95% CI	0.18–1.24	0.04–0.58	0.11–1.13
		χ^2 / p	2.15 / 0.17	7.76 / 0.005	2.92 / 0.09
	Homo-/heterozygous with allele 177 vs. others	OR	0.47	0.16	0.35
		95% CI	0.16–1.43	0.04–0.64	0.11–1.16

		χ^2 / p	1.48 / 0.22	6.25 / 0.01	2.69 / 0.10
A113	Allele 218 vs. others	OR	1.38	2.38	1.72
		95% CI	0.74–2.57	1.18–4.80	1.04–1.39
		χ^2 / p	0.90 / 0.34	6.12 / 0.01	4.91 / 0.03
Ap243	Allele 263 vs. others	OR	1.25	0.21	0.17
		95% CI	0.27–2.83	0.06–0.75	0.06–0.53
		χ^2 / p	0.17 / 0.68	5.51 / 0.02	10.99 / 0.0009
	Homo-/heterozygous with allele 263 vs. others	OR	1.00	0.18	0.19
		95% CI	0.37–2.74	0.05–0.73	0.06–0.51
		χ^2 / p	0.05 / 0.82	5.19 / 0.02	8.22 / 0.004
A024	Allele 92 vs. others	OR	0.77	0.56	0.73
		95% CI	0.38–1.53	0.26–1.17	0.46–1.15
		χ^2 / p	0.41 / 0.52	2.25 / 0.13	1.80 / 0.18
	Allele 100 vs. others	OR	1.21	1.99	1.64
		95% CI	0.51–3.00	0.80–5.10	0.96–2.16
		χ^2 / p	0.07 / 0.79	2.00 / 0.16	3.24 / 0.07
A007	Allele 104 vs. others	OR	1.35	2.18	1.62
		95% CI	0.46–4.19	0.69–7.32	0.78–3.32
		χ^2 / p	0.12 / 0.73	1.47 / 0.23	1.53 / 0.22
Ap049	Allele 120 vs. others	OR	0.72	0.31	0.44
		95% CI	0.30–1.76	0.09–1.04	0.16–1.15
		χ^2 / p	0.34 / 0.56	3.57 / 0.06	2.67 / 0.10
SV185	Allele 269 vs. others	OR	0.61	0.44	0.72
		95% CI	0.31–1.18	0.21–0.93	0.44–1.20
		χ^2 / p	2.00 / 0.16	4.68 / 0.03	1.43 / 0.23

OR—odds ratio; 95% CI—confidence interval limits; χ^2/p—chi-square test and significance level, df = 1. Alleles showing statistically significant OR values are marked in bold.

For AC117, A113 Ap243, A024, A007, Ap049, and SV185, significant differences in allele and/or genotype distributions were observed among infection groups. Nevertheless, only a subset of allelic variants from these loci demonstrated strong associations with Nosema status.

Based on OR analysis, alleles “177” (AC117), “263” (Ap243), and “269” (SV185) appeared to act as protective factors, reducing the likelihood of infection.

The frequency of genotypes carrying allele “177” at AC117 showed a steady decline along the infection gradient: 25.9% in uninfected bees, 14.1% in mildly infected, and only 4.9% in highly infected individuals. The difference between uninfected and highly infected groups reached statistical significance ($\chi^2 = 16.61$, df = 2, $p < 0.01$).

For Ap243, the proportion of genotypes containing allele “263” in heavily infected bees (11.1%) was significantly lower compared with both uninfected bees (43.3%; $\chi^2 = 13.45$, df = 6, $p < 0.05$) and mildly infected bees (43.5%; $\chi^2 = 18.86$, df = 7, $p < 0.01$).

At SV185, no statistically significant genotype-level differences were found ($p > 0.05$). On the other hand, allele “218” of A113 was associated with higher susceptibility to nosemosis, though no genotype-level significance was recorded.

Discussion

This work assessed the genetic variability of *A. m. mellifera* colonies that differed in their levels of *Nosema* infection, with the goal of identifying links between microsatellite genotypes and susceptibility to nosemosis. The data demonstrated that certain alleles were correlated with infection resistance among Siberian *A. m. mellifera* bees. Specifically, alleles “177” of the AC117 locus, “263” of the Ap243 locus, and “269” of the SV185 locus appeared to lessen infection likelihood.

The study represents an initial attempt to explore genetic indicators connected with *Nosema* infection or resistance in honey bees. Whether these findings reflect consistent biological mechanisms or occurred by chance remains

uncertain. To clarify this, future investigations should include broader sample sets, incorporate other *Apis mellifera* subspecies, and evaluate the influence of various microsatellite regions on nosemosis tolerance.

Earlier, in collaboration with T. Kireeva (Tomsk State University; unpublished results) [78], we analyzed microsatellite associations in *A. m. carpathica*. In that dataset, the Ap243 locus also exhibited marked statistical variation between *Nosema*-infected and uninfected bees ($\chi^2 = 22.93$, df = 7, $p < 0.01$). Alleles "253" and "256" were responsible for the group differences ($\chi^2 = 9.69$, df = 1, $p < 0.01$; and $\chi^2 = 7.03$, df = 1, $p < 0.01$). Odds ratio analysis indicated that allele "256" conferred protection (OR = 0.29, 95% CI—0.10–0.84, χ^2/p—6.87/0.0088, χ^2-Yeats/p—5.57/0.0182), whereas allele "253" raised infection risk (OR = 3.57, 95% CI—1.53–8.40, χ^2/p—11.10/0.00086, χ^2-Yeats/p—9.77/0.0018).

Hence, the Ap243 locus on chromosome 1 (group 1.1) was common to both *A. m. mellifera* and *A. m. carpathica* and appears to play a role in either infection occurrence or defense. Despite the shared locus, the alleles involved differed. In *A. m. mellifera*, the allele "263" likely offered protection, and significant genotype-level distinctions were recorded between *Nosema*-positive high, *Nosema*-positive low, and uninfected bees. Conversely, in *A. m. carpathica*, two alleles ("256" and "253") were significantly different between infected and healthy bees, with "256" likely beneficial and "253" disease-related.

The A024 locus also merits attention. Although in *A. m. mellifera* no allele of this locus showed statistical connection with *Nosema* **(Table 2)**, in *A. m. carpathica* the "90" allele seemed linked to resistance (OR = 0.09, 95% CI—0.04–0.023, χ^2/p—39.94/0.0000000, χ^2-Yeats/p—37.93/0.0000000).

Distinct microsatellite regions and alleles connected to infection or resistance were therefore found for the two bee subspecies. These differences could result from their differing natural resistance, ecological conditions, or habitat-specific influences such as geography, temperature, and diet [58, 79-82]. Moreover, such variation may depend on the genomic architecture of the chromosomal segment housing the QTL. It is possible that not the identified locus itself, but another closely linked site, determines resistance. Thus, in separate subspecies, distinct alleles may be inherited together with a favorable genetic variant.

Comparable findings have been noted in studies searching for QTLs associated with hygienic traits and *Varroa* resistance in bees [14, 20-22, 31]. Research has revealed more than 20 candidate chromosomal regions within linkage groups 2, 3, 4, 5, 6, 7, 9, 10, 13, 15, 16, and 22. Using RAPD markers, Lapidge *et al*. (2002) identified seven potential QTLs related to hygienic behavior [22]. Microsatellite analyses by Oxley *et al*. (2010) identified three significant and three suggestive QTLs influencing worker behavior [31], while Behrens *et al*. (2011) discovered three QTLs connected to reduced mite reproduction in *Varroa*-tolerant Swiss lineages [20]. Similarly, Spötter *et al*. detected six SNPs showing strong genome-wide correlation with *Varroa*-related hygiene traits [14]. These investigations did not report identical QTLs. For instance, Oxley *et al*. (2010) and Behrens *et al*. (2011) both found two QTLs on chromosome 9, though in separate regions [20, 31]. Likewise, Tsuruda *et al*. (2012) noted a major QTL on a different segment of the same chromosome using an SNP-Chip approach [21]. Such variation in mapped loci may arise from differing biological materials (freeze-killed or infested brood, or adult workers), methodological diversity, and marker systems (RAPD, microsatellite, SNP). Divergent genetic maps—constructed with different marker types—may also contribute. Variation in the bee subspecies studied further explains these inconsistencies. For instance, Oxley *et al*. (2010) and Spötter *et al*. (2016) both located QTLs on chromosomes 2 and 5, but on opposite ends [14, 31]. The observed discrepancies likely stem from the use of distinct biological materials: Oxley *et al*. used freeze-killed brood [31], while Spötter *et al*. examined *A. m. ligustica* and *A. m. carnica* [14].

When comparing the QTLs related to *N. ceranae* infection tolerance described by Huang *et al*. [35] with the trait-associated genomic regions identified in the current study, no overlap was observed. Huang and colleagues reported four significant QTLs on chromosomes 3, 10, 6, and 14 that were correlated with reduced *Nosema* spore load, collectively accounting for 20.4% of the total spore load variation in the Danish *Nosema*-resistant honey bee strain. Among these, the QTL located on chromosome 14 explained 7.7% of the total variance and was proposed as a potential determinant of resistance to nosemosis in that population. Within this genomic region, the candidate gene Aubergine (Aub) showed notably higher expression in drones exhibiting low spore loads compared to those with high levels of infection [35].

In the current dataset, the dark forest bee displayed associations between nosemosis resistance and three microsatellite loci: Ap243 (chromosome 1), SV185 (chromosome 5), and AC117 (chromosome 12). Particularly noteworthy is that on chromosome region 1.1, the Ap243 locus lies close to the microRNA ame-miR-2b. Previous studies have demonstrated that host microRNAs can actively respond to *N. ceranae* invasion [36]. During

infection, 17 microRNAs were differentially expressed in honey bees, targeting more than 400 predicted genes linked to ion binding, signal transduction, nuclear processes, membrane transport, and DNA binding. The miRNA ame-miR-2b is of special relevance since the expression of 11 out of 27 enzyme genes was significantly correlated with its activity [36]. Moreover, this same chromosomal region also harbors a QTL previously associated with Varroa sensitive hygiene performance [21]. This locus contains over 30 potential candidate genes [21], including the puromycin-sensitive aminopeptidase**,** which participates in proteolytic processes crucial for cell viability and growth [83]; selenoprotein F, an endoplasmic reticulum protein regulated under stress conditions [84]; as well as genes coding for transcription and splicing regulators.

Considering that *Nosema* spp. are intracellular microsporidian parasites [37], the cell wall integrity and stress response component 1 also deserves attention. In *Schizosaccharomyces pombe*, its homolog—wsc1—is known to mediate cell surface receptor signaling, intracellular signaling cascades, and the regulation of cell wall construction and remodeling [85].

Although many QTLs linked to honey bee disease resistance have been mapped, the variation in this trait is believed to be under the influence of only a limited number of loci. For instance, hygienic behavior exhibits a strong genetic foundation, though environmental influences may also play a role [14]. Consequently, identifying and characterizing the allelic variants responsible for enhanced disease resistance—and subsequently applying this knowledge in selective breeding—offers a promising avenue for the development of more resilient bee populations.

Conclusion

This study identified relationships between specific microsatellite loci and *Nosema* infection in honey bees. In the dark forest bee, promising genetic indicators of resistance were detected, namely allele “177” at AC117, allele “263” at Ap243, and allele “269” at SV185. Nonetheless, variations in loci and allele patterns determining nosemosis resistance across subspecies or breeds remain unresolved. Therefore, further studies involving both the same bee subspecies maintained in different regions and other genetic lineages are required. Even so, the current findings suggest that these markers may already serve as predictors for assessing the risk of nosemosis, provided that their diagnostic value is evaluated separately for each specific bee subspecies or breed.

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