Original Article

Infection Rate of *Vairimorpha* spp. in Honey Bees (*Apis mellifera*) in Northern Iran Using Microscopic and Molecular Methods

Sahel Molaei Baghal[1], Seyed Reza Miraei Ashtiani[1*], Sedigheh Nabian[2*], Mahsa Shahbakhsh[2], Alieza Arab[1], Ali Ahmadi[2] Abbas Gerami Sadeghian[2]

1. Department of Animal Science, Faculty of Agriculture, University College of Agriculture & Natural Resources, University of Tehran, Karaj, Iran.
2. Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

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ABSTRACT

Background: Honey bees (*Apis mellifera*) are critical pollinators, significantly contributing to agricultural productivity and biodiversity. In recent years, however, there has been a marked decline in honey bee populations, primarily due to habitat loss, climate change, and disease.

Objectives: This study investigates the infection rates of *Vairimorpha apis* (formerly *Nosema apis*) and *Vairimorpha ceranae* (formerly *Nosema ceranae*) in honey bee colonies in northern Iran amid growing concerns about declining bee health.

Methods: A total of 373 samples were collected between September 2021 and January 2022. Initially, the samples were examined using microscopy. Subsequently, the positive samples were analyzed using molecular techniques.

Results: A total of 97 apiaries across Gilan, Mazandaran, and Golestan provinces were monitored. The infection rate of *Vairimorpha* spp. was 65.97%, and only *V. ceranae* was identified as the causative agent of nosemosis, as confirmed by microscopic and molecular analyses. Of the 485 samples examined, 41.03% tested positive for *N. ceranae*, indicating a significant infection rate across the studied regions.

Conclusion: This study highlights the pressing issue of *Vairimorpha* infection in Iranian honey bee colonies and underscores the need for continued surveillance and research to address the factors driving declines in honey bee health. The findings provide critical insights into the epidemiology of nosemosis and can facilitate the development of informed management strategies to safeguard honey bee populations.

Keywords: *Vairimorha* spp., Honeybee, Microscopy, PCR, Iran

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*** Corresponding Authors:**
Sedigheh Nabian, Professor.
Address: *Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*
E-mail: *nabian@ut.ac.ir*

Seyed Reza Miraei Ashtiani, Professor.
Address: *Department of Animal Science, Faculty of Agriculture, University College of Agriculture & Natural Resources, University of Tehran, Karaj, Iran.*
E-mail: *ashtiani@ut.ac.ir*

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Introduction

Honey bees (*Apis mellifera*) are vital components of our ecosystems, serving as highly efficient pollinators for both food crops and wild plants. Their presence is crucial for maintaining agricultural productivity and ecosystem health. However, in recent years, the populations of various pollinators, especially honey bees, have significantly declined (Ütük et al., 2019). This decline poses a serious threat to plant species and biodiversity across many regions of the world (Mayack et al., 2022), prompting an increase in research focused on bee health.

Recent reviews have highlighted that significant declines in insect populations, including honey bees, predominantly occur in Europe due to both biotic and abiotic factors, such as agricultural intensification, climate change, and invasive species (Vega & Kaya, 2012). Among these factors, infections from parasites and pathogens are likely the leading contributors to colony mortality (Whitaker et al., 2011).

Recent discussions surrounding the classification of microsporidian species have led to significant debate within the scientific community. Tokarev et al. attempted to redefine 17 microsporidian species, including those in the genera *Nosema*, *Vairimorpha*, *Rugispora*, and *Oligosporidium*, based on phylogenetic analyses utilizing two genetic markers (SSU rRNA and RPB1) (Tokarev et al., 2020). However, this reclassification has been critiqued for several methodological flaws that could invalidate the proposed distinctions and may ultimately lead to the synonymization of *Vairimorpha* within *Nosema*. These ongoing debates underscore the importance of rigorous taxonomic reviews in understanding the ecology and impact of these pathogens on honey bee health.

Two microsporidian species, *V. apis* and *V. ceranae*, are obligate intracellular parasites found worldwide that cause nosemosis in honey bees. While both *V. apis* and *V. ceranae* infect honey bees, *V. ceranae* has been shown to outcompete *N. apis* geographically (Ostroverkhova et al., 2020). Severe infections with *V. ceranae* can lead to increased mortality rates among bees and are associated with significant colony losses (Martín-Hernández et al., 2018). Moreover, *V. ceranae* is linked to numerous detrimental physiological effects, including suppressed immune function, altered foraging behavior, disrupted pheromone and hormone production, and impaired lipid synthesis (Ostroverkhova et al., 2020).

This pathogenic agent replicates in the epithelial cells of the midgut, disrupting normal digestive functions, which can lead to malnutrition, physiological aging, and reduced lifespan in affected bees. As a result, these populations often experience declines in honey production. *V. ceranae* was first identified in 1994 in its primary host, *Apis cerana* (Fries et al., 1996). In 2006, it was identified as the primary cause of nosemosis in *A. mellifera* in Spain (Klee et al., 2017). Since then, *V. ceranae* has been detected in various regions, including Europe, Asia, and the Americas, where it has been linked to the recent phenomenon of colony decline (Goblirsch, 2018).

In Iran, various microsporidian species have been reported in honey bees and silkworms (Nabian et al., 2011; Ghoyounchi et al., 2017; Bagheri et al., 2023). A study in Gilan Province reported a prevalence of *V. apis* infection ranging from 30% to 70% (Nabian, 2017). Following reports of colony collapse disorder (CCD) in Iran, comprehensive surveys across multiple provinces confirmed the presence of *V. ceranae* using molecular methods (Nabian, 2017). Further studies utilizing molecular techniques have consistently demonstrated the presence of *V. ceranae* in many Iranian apiaries (Nabian, 2017).

Recent research indicates significant losses in bee colonies in northern Iran, with the *Vairimorpha* parasite recognized as one of the primary contributors to these declines. Therefore, the aim of the present study was to investigate the rate of *Vairimorpha* infection in honey bee populations in the three provinces of Gilan, Golestan, and Mazandaran and determine the dominant species in this region.

Materials and Methods

Ecological and geographical characteristics of the region

This study investigated *Vairimorpha* infection in honey bees across several provinces in northern Iran, specifically Gilan, Mazandaran, and Golestan. These provinces are situated within the Hyrcanian forests, which form a verdant belt of predominantly temperate deciduous ecosystems along the northern slopes of the Alborz Mountain Range, bordering the southern shores of the Caspian Sea. According to data from the Forests, Rangelands, and Watershed Organization (FRWO), the Caspian Hyrcanian mixed forests span approximately 800 kilometers in length and 110 kilometers in width, encompassing a total area of 1.85 million hectares. These forests represent about 15% of Iran's total forest cover and 1.1% of the country's land area (Sagheb Talebi et al., 2014).

Sample collection

To assess the prevalence of *Vairimorpha* spp. in the northern provinces of Iran (Hyrcania region, which includes forest, mountain, and plain ecozones), we monitored 97 apiaries for *Nosema* infection from September 2021 to January 2022. In each apiary, five colonies were randomly selected as representative samples. In apiaries with fewer than five hives, all beehives were included in the sampling process.

Samples from each hive were transferred to containers with ventilation apertures. Each container held at least 100 live bees and was labeled with the beekeeper's name, phone number, province, city, sampling date, and hive number for identification. The samples were stored at -20 ˚C until DNA extraction.

Microscopy examination

Upon arrival at the laboratory, the collected bee samples were immediately stored at 20 ˚C. The abdomens of 20 honey bees were ground in 5 mL of water, followed by the addition of 15 mL of water to achieve a final volume of 20 mL (1 mL per bee). This solution was then filtered through two layers of muslin.

Once the solution was homogeneous, one drop was placed in a hemocytometer chamber for counting. The *Vairimorpha* spores were observed using a light microscope at ×400 magnification (Shirzadi & Razmi, 2021). Spores were categorized based on their counts as follows: 1-20 spores indicated weak infection (+), 20-50 spores indicated moderate infection (++), 50-100 spores indicated high infection (+++), and counts exceeding 100 spores indicated severe infection (++++) (Fries et al., 2013).

Molecular analysis

Total genomic DNA was extracted using a DNA extraction kit (MBST, Iran) according to the manufacturer's instructions (Research Institute Molecular Biological System Transfer, 2021). All DNA samples were stored at -20 ˚C until further use. Multiplex polymerase chain reaction (PCR) was performed using primers designed based on the 16S RNA. The primers utilized for *V. apis* were 321 APIS FOR (5′-GGGGGCATGTCTTTGACGTACTATGTA-3′) and 321 APIS REV (5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′). For *V. ceranae*, the primers were 218 MITOC FOR (5′-CGGCGACGATGTGATATGAAAATATTAA-3′) and 218 MITOC REV (5′-CCCGGTCATTCTCAAACAAAAAACCG-3′) (Martín-Hernández et al., 2007).

The DNA amplification parameters included an initial activation step at 94 ˚C for 2 minutes, followed by 10 cycles of 15 seconds at 94 ˚C, 30 seconds at 61.8 ˚C, and 45 seconds at 72 ˚C, and then 20 cycles of 15 seconds at 94 ˚C, 30 seconds at 61.8 ˚C, and 50 seconds at 72 ˚C, concluding with a final extension at 72 ˚C for 7 minutes. The PCR products were visualized on a 1.5% agarose TAE gel, with bands appearing at 321 bp for *V. apis* and at 218–219 bp for *V. ceranae*.

Sequence analysis

Fifty positive samples were sequenced to confirm the species of the amplified *Vairimorpha* DNA. The nucleotide sequences obtained were compared with all available *V. apis* and *V. ceranae* sequences in the GenBank™ database using the BLAST tool.

Statistical analyses

Statistical analyses were conducted using SPSS version 23.0. The chi-square test was employed to assess differences in *Vairimorpha* distribution across various regions, with a significance level set at 0.05.

Results

Honey bee samples collected from 97 apiaries across several regions of the Hyrcanian ecozone between September 2021 and January 2022 were examined for the presence of *Vairimorpha* infections. *Vairimorpha* spp. were detected in 64 out of the 97 monitored apiaries (65.97%). At the colony level, microscopic examination of all 485 samples revealed that 154 were infected (41.03%).

Microscopic examination of the 373 samples revealed that 154 were infected with nosemosis, indicating an infection rate of 41.3% (Figure 1). The chi-square test results indicated a χ^2 value of 21.000 with 18 degrees of freedom. The P-value was 0.27, suggesting that there were no statistically significant differences in *Nosema* distribution among the different regions (P>0.05).

Molecular analysis identified *V. ceranae* as the sole species responsible for nosemosis in northern Iran, as no cases of *V. apis* were detected. PCR results confirmed the presence of *V. ceranae*, showing a specific amplicon at 218 bp (Figure 2). BLAST analysis revealed that our query sequences were identical to those of various geographical isolates of *V. ceranae*.

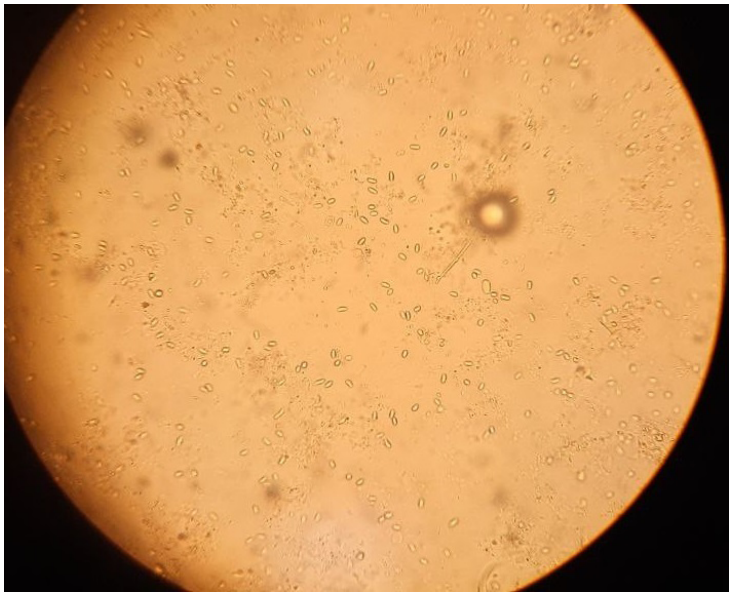


Figure 1. Presence of *Vairimorpha* spores (wet mount, 400X) in macerated abdominal suspension of adult honey bees

Discussion

V. ceranae and *V. apis* differ in environmental tolerances and epidemiology. *V. ceranae* is particularly sensitive to freezing, whereas *V. apis* can endure storage at low temperatures, a difference that shapes their geographic and seasonal distributions (Mazur & Gajda, 2022). In warmer regions, where *V. ceranae* tends to predominate, infections are often more prevalent, whereas *V. apis* is more commonly associated with cooler climates (Hatija et al., 2022; Forsgren & Fries, 2013). In recent years, *V. ceranae* has largely supplanted *V. apis*, a shift linked, at least in part, to changing climatic conditions and associated ecological pressures (Martín-Hernández et al., 2018; Mazur & Gajda, 2022). The epidemiological consequences of this replacement have been implicated in colony losses in several Mediterranean countries and other temperate regions (Topolska et al., 2013).

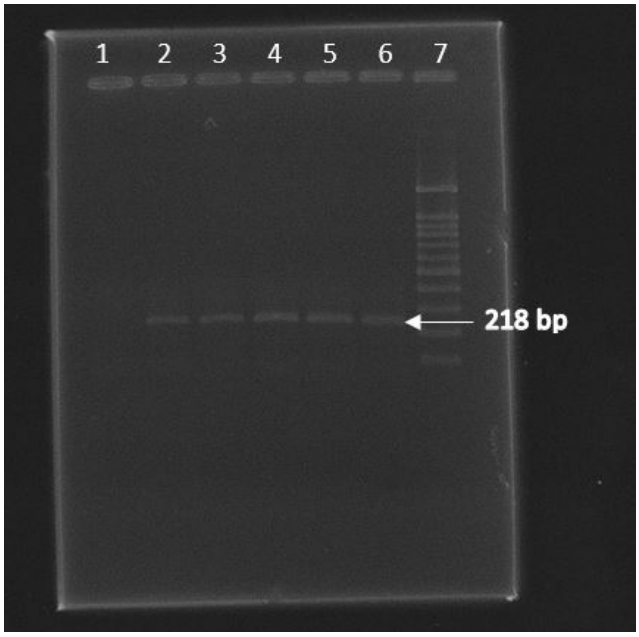


Figure 2. Agarose gel electrophoresis of the 16S samples of *Vairimorpha* (formerly *Nosema*)

Note: The 100-bp ladder is shown in lane 7; lanes 2–6 show *V. ceranae*, and lane 1 is the negative control.

Table 1. Infection rates of *Nosema* infections in different provinces of the Hyrcania ecozone

Province	No. of Samples	No. of Positive Microscopic Results	Infection Rate (%)
Gilan	160	75	46.87
Golestan	190	72	37.89
Mazandaran	135	52	38.51
Total	485	199	41.03

Vairimorpha (*Nosema*) infections in honey bees show marked heterogeneity across regions and seasons, with notable shifts in species composition over time. Our study conducted in Hyrcania (northern Iran) found that 41.03% of honey bee samples were infected with *Vairimorpha*, and molecular analyses identified *V. ceranae* as the sole species responsible for nosemosis in these apiaries. This finding aligns with multiple reports from other regions where *V. ceranae* predominates and, in some cases, replaces *V. apis*. Khezri et al. (2018) reported a 34% infection rate in the Kurdistan Province of Iran using molecular methods. In Romania, Medrele et al. found a 55% infection rate in apiaries, with *V. ceranae* as the sole species identified (Medrele et al., 2018). Similarly, Ütük et al. reported a 41.6% infection rate in Azerbaijan and identified *V. ceranae* as the only species detected (Ütük et al., 2019). In Brazil, a study by Lage et al. found that 62.34% of samples collected in Bahia State were infected with *V. ceranae* (Lage et al., 2022). Lotfi et al. reported that 85% of the apiaries in East Azerbaijan Province were contaminated with *V. apis* (Lotfi et al., 2009). For the first time, Nabian et al. in 2011 diagnosed *V. ceranae* in Iran. In this study, using a molecular method, *V. ceranae* was identified in honey bees in Mazandaran Province (Nabian et al., 2011).

Modirrousta et al. demonstrated using the multiplex PCR method that *Vairimorpha* in the provinces of Alborz, East Azerbaijan, Qazvin, Gilan, and Tehran consisted exclusively of *V. ceranae* (Modirrousta et al. 2014; Modirrousta et al. 2014).

Additionally, Mohammadian et al. investigated the prevalence of *Nosema* in different climatic regions of Iran in a cross-sectional study conducted in 2016. The results showed that *V. ceranae* infection was present in all climatic regions studied. The prevalence of *V. ceranae* was 24.2% (10.1-10.5). *V. apis* infection was not observed in the samples, either as a single infection or as a co-infection. Based on the PCR results, the prevalence of *V. ceranae* was 19.8% (40.24-45%) in humid climates, (10.7-77.19) in semi-humid climates, (48.5% - 80.72-45) in very humid climates, (43.2% - 50.44% - 70.94) in dry climates, (40.20-25.47) in semi-arid climates, and (42% - 90%) in Mediterranean climates (42% - 90%). Infection prevalence differed significantly among the country's climatic regions ($P<0.005$). According to this study's findings, *V. ceranae* was the only *Vairimorpha* species with a wide geographic distribution in Iranian apiaries. Climate may affect the prevalence of this parasite (Mohammadian et al. 2018).

Additionally, Razmaraii and et al. reported a *V. ceranae* prevalence of 58.1% using microscopic methods and 67.1% using molecular methods in East Azerbaijan Province (Razmaraii et al. 2017).

Moeini et al. reported that *V. ceranae* infection in Urmia Province reached 58.2% using multiplex PCR (Moeini et al. 2022). Mefthi et al. investigated infection with *Vairimorpha* species in migratory apiaries in Hormozgan Province using microscopic and molecular methods. Microscopic examination showed that 38.2% of the apiaries were positive for *Vairimorpha* species spores. Molecular examination found that 39.2% of the apiaries were infected with *V. ceranae*, and no samples were positive for *V. apis* (Metahi et al. 2023).

Despite these insights, our study had several limitations. Sampling could not be conducted at the end of summer, and delays in sample shipment, inadequate packaging, and failure to maintain optimal storage conditions disrupted the research process. Therefore, future studies should focus on additional ecozones and assess the seasonal prevalence of this disease. The results should be interpreted in the context of regional weather conditions and seasonal climatic variations.

Conclusion

The findings of this study confirm the presence of *Vairimorpha* infection in northern Iran. Molecular analyses identified all isolates as *V. ceranae*, with no evidence of *V. apis* detected in the surveyed regions. These findings can inform the development of targeted prevention and control strategies.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and methodology: Seyed Reza Miraei Ashtiani and Mahsa Shahbakhsh; Investigation: Sahel Molaei Baghal, Mahsa Shahbakhsh, Abbas Gerami Sadeghian, and Ali Ahmadi; Sample collection: Ali Ahmadi and Alireza Arab; Writing the original draft: Mahsa Shahbakhsh; Review and editing: Sedigheh Nabian.

Conflict of interest

The authors declared no conflict of interest.

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