Citation/Atıf: Kartal S, Tunca Rİ, Özgül O, Karabağ K, Koç H. 2021. Microscopic and molecular detection of *Nosema sp.* in the Southwest Aegean region (Güneybatı Ege Bölgesinde Nosema Türlerinin Mikroskobik ve Moleküler Yöntemlerle Belirlenmesi). U. Arı D./U. Bee J. 21: 8-20, DOI: 10.31467/uluaricilik.880380

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

MICROSCOPIC AND MOLECULAR DETECTION OF NOSEMA SP. IN THE SOUTHWEST AEGEAN REGION

Güneybatı Ege Bölgesi'nde Nosema Türlerinin Mikroskobik ve Moleküler Olarak Belirlenmesi

Serengül KARTAL[1], Rahşan İVGİN TUNCA[2], Okan ÖZGÜL[2], Kemal KARABAĞ[3], Hasan KOÇ[4]

[1]Muğla Sıtkı Koçman University, Graduate School of Natural and Applied Sciences, 48000, Kötekli, Muğla, TURKEY, ORCID No.: 0000-0003-1751-8976, E-posta: kartalserengul@gmail.com.

[2] Muğla Sıtkı Koçman University, Ula Ali Koçman Vocational School, 48640, Ula, Muğla, TURKEY, ORCID No.: 0000-0003-0745-6732, Yazışma Yazarı/Corresponding author: E-posta: rivgin@gmail.com

[3] Akdeniz University, Agriculture Faculty, Dept. of Animal Biotechnology, 07058, Antalya, TURKEY, ORCID No.: 0000-0002-4516-6480, E-posta: karabag@akdeniz.edu.tr.

[4] Muğla Sıtkı Koçman University, Department of Biology, 48000, Kötekli, Muğla, TURKEY, ORCID No.: 0000-0002-2560-4527, E-posta: khasan@mu.edu.tr.

Geliş Tarihi / Received: 15.02.2021 Kabul Tarihi / Accepted: 24.04.2021 DOI: 10.31467/uluaricilik.880380

ABSTRACT

Beekeeping, performed in many parts of the world, has a very large place in the world trade market with bee products such as wax, bee venom, propolis and royal jelly, especially honey production. However, honey bee diseases are quite common and restricted the production of bee products. One of the most important of these diseases, Nosema, is caused by spores in intestinal epithelium cells of the honeybee. *Nosema apis* and *Nosema ceranae* are the factors of this disease and also common in our country. These two species can be distinguished from each other by molecular diagnostic methods. In this study, materials collected from 152 apiaries located in 13 districts of Muğla province and 62 water sources close to these apiaries. The spores were counted using Thoma lame under light microscope. DNA isolation was carried out from spore positive samples. 218MITOC FOR-REV and 321APIS FOR-REV primers were used to figure out the *N. apis* and *N. ceranae* species. After DNA sequence analysis of the obtained amplifications, it was determined that all samples formed 3 haplotypes according to studied sequences for the first time. In Muğla region, the presence of only *N. ceranae* as a disease agent was determined and the prevalence of the disease was detected at a rate of 71.53±6.02%. Moreover, blast analysis showed that the *N. ceranae* sequence detected high similarity (94-100 %) with the previously reported in Lebanon, France, Morocco and Thailand samples.

Keywords: *N. apis, N. ceranae,* molecular detection, Haplotype, Muğla, Turkey

ÖZET

Dünya'nın pek çok yerinde hayvansal üretim faaliyeti olarak yapılan arıcılık, başta bal üretimi olmak üzere bal mumu, arı zehri, propolis, arı sütü gibi arı ürünleri ile de dünya ticaret pazarında oldukça geniş bir yere sahiptir. Ancak, arıcılıktan elde edilecek verimi kısıtlayan bal arısı hastalıkları oldukça yaygınlaşmış durumdadır. Bu hastalıkların en önemlilerinden biri olan Nosema, bal arısının bağırsak

epitelyum hücrelerinde sporların neden olduğu hastalıktır. Ülkemizde de yaygın bulunan bu hastalığın etmeni olarak *Nosema apis* ve *Nosema ceranae* gösterilmektedir. Bu iki tür birbirlerinden en iyi şekilde moleküler tanı yöntemleri ile ayırt edilebilmektedir. Bu çalışmada, Muğla ilinin 13 farklı lokasyonunda bulunan 152 arılıktan ve bu arılıklara yakın 62 su kaynağından alınan örneklerde Nosema sporları ışık mikroskobu altında Thoma lamı kullanılarak spor sayımı yapılmıştır. Nosema sporu gözlemlenen örneklerden DNA izolasyonları gerçekleştirilmiştir. Nosema tür taraması için 218MITOC FOR- REV ve 321APIS FOR-REV primerleri kullanılarak ilgili gen bölgeleri çoğaltılmıştır. Yapılan network analizinde bu gen bölgelerine göre ilk kez 3 haplotipi belirlenmiştir. Muğla yöresinde Nosema hastalığı yaygınlığı %71,53±6,02 oranında tespit edilmiş ve hastalık etmeni olarak sadece *N. ceranae'*nın varlığı belirlenmiştir. Ayrıca, blast analizi, daha önce Lübnan, Fransa, Fas ve Tayland ülkelerinden bildirilen *N. ceranae* örnekleri ile yüksek benzerlik (%94-100) tespit edilmiştir.

Anahtar kelimeler: *N. apis*, *N. ceranae* moleküler tespit, Haplotip, Muğla, Turkiye

GENİŞLETİLMİŞ ÖZET

Amaç: Bal arıları ekonomik ve biyolojik yönden oldukça önemlidir. Bal arıları bal, propolis, arı sütü, polen, bal mumu, arı zehri gibi arı ürünleri sayesinde dünya pazarında önemli yer almaktadır. Arıcılık, Dünya'nın hemen hemen her yerinde yapılan tarımsal bir faaliyettir. Arıcılık faaliyetlerini engelleyen en önemli nedenlerden biri de arı zararlı ve hastalıklarıdır. Nosemosis, ergin balarılarında oldukça yaygın görülen *Nosema apis* (Zander, 1909) ve *Nosema ceranae* (Fries et al., 1996) adlı mikrosporidiaların neden olduğu bir hastalıktır. Hastalık kolonilerde genel olarak koloni performansını etkiler ve populasyon sayısının düşmesine neden olarak kolooninin yok olmasına sebebiyet vermektedir. Farklı türdeki Nosema sporlarının birbirlerinden ayırt etmede en etkili yolu moleküler tanı yöntemleridir. Bu çalışmada Muğla ili genelinden toplanan bal arısı örnekleri ve kovanlara yakın su kaynaklarından alınan su numunelerinde Nosema hastalığının mikroskobik ve moleküler teşhisi yapılarak Dünya genelinde ciddi koloni kayıplarına neden olan bu hastalığın Muğla yöresindeki varlığı ve yaygınlığının belirlenmesi hedeflenmiştir.

Yöntem: Bal arısı örnekleri, Muğla ilini temsilen 13 ilçeden belirlenen 152 arılıktan, her arılıktan tesadüfi olarak belirlenmiş ortalama 20 kovanın girişinden yaklaşık 100 adet olacak şekilde arı örneği toplanmıştır. Su örnekleri arı kovanlarına yakın 62 su kaynağından (Bunlardan 12 tanesi arılıkların içindeki arıcılar tarafından yerleştirilmiş olan suluklar, geri kalanları ise arılıklara yakın olan akarsular, su yolları ve çeşmelerdir) en az 50 ml olacak şekilde su numuneleri alınmıştır. Arazi çalışmaları ilkbahar Nisan-Mayıs ve sonbahar Ekim-Kasım aylarında 2017 yılında tamamlandı. Laboratuvara getirilen arı örneklerinden Dünya Hayvan Sağlığı Örgütü (OIE) uygulama kılavuzun göre homojenatlar hazırlandı. Sporların tespiti, sayımı ve hesaplanması 400x büyütmeli mikroskop altında thoma lamı kullanılarak yapılmıştır. 13 ilçeden ortalama en yüksek spor sayısına sahip 3 arılıktan alınan örneklerde ticari izolasyon kiti (PureLinkGenomic DNA Mini Kit) kullanılarak DNA izolasyonları yapıldı. 218MITOC For-Rev ve 321APIS For-Rev primerleri, ilgili gen bölgeleri Polimeraz Zincir Reaksiyonu (PZR) ile çoğaltıldı. Sekans analizine toplam 35 PCR sonucu gönderildi ancak 30 tanesinin sekans sonucu değerlendirilebilir bulundu. Değerlendirilen sonuçların MEGA 6.0 programında sekans dizileri, SplitsTree programı kullanılarak filogenetik ağaç görüntüsü ve Network programı kullanılarak haplotip belirlemesi yapıldı. BLAST veri tabanı üzerinden sekans benzerlikleri analizi yapıldı.

Sonuç: Muğla yöresinde Nosema hastalığı yaygınlığı %71,53±6.02 oranında tespit edilmiş ve toplanan 62 su örneğinden sadece 5 tanesinde Nosema sporlarına rastlanmıştır. Hastalık etmeni olarak sadece *N. ceranae'*nın varlığı belirlenmiştir. PZR sonrasında DNA dizisine gönderilen 35 örneğin yalnızca 30'unun dizi sonucu değerlendirilebildi. BLAST analizi sonucunda örnekler Lübnan, Fransa, Fas ve Tayland örneklerinde belirlenen *N. ceranae* dizileri ile%94-100 benzerlik göstermiştir. Hit dizileri MEGA 6 programında hizalandı. Yapılan network analizinde bu gen bölgelerine göre 3 haplotipi belirlenmiştir. Filogenetik ağaca göre Ula ve out grup farklı dal üzerinde yer alırken, Muğla'dan alınan diğer örnekler Muğla'dan gelen diğer gruplar ise diğer ana dal üzerinde yer almaktadır.

INTRODUCTION

Honeybees (*Apis mellifera* L.) provide not only bee products such as honey, propolis, royal jelly, pollen, bee wax, bee venom to be placed in the market by ensuring the production of World trade (Özbek 2002) but also pollinating the wild flora and industrial crops (Van Engelsdorf and Meixner 2010). However, bee diseases and pests affect beekeeping activities and the quality of the products obtained from beekeeping. Bee diseases and pests spread all over the world in a short time because of the trading of the bees, bee products and beekeeping materials between countries (Öztürk 2001). Also, migratory beekeeping activities are an important factor in the rapid spread of honey bee diseases and pests within the country (Gülpınar 2005). Because of these reasons, honey bee diseases and pests are one of the most important factors that slowing the progress of beekeeping in our country and decrease the efficiency of production (Doğaroğlu 1999). Nosemosis is a disease caused by microsporidia called *Nosema apis* and *Nosema ceranae*, which are quite common in adult honeybees. *N. apis* was first described by Zander (1909) and has a worldwide distribution (Matheson1996). *N. ceranae* was reported in 1996 in the Asian honey bee, *Apis cerana* (Fries et al.1996). Later, Higes et al. (2006) mentioned that *A. mellifera* in Europe was infected by *N. ceranae*.

Shortly after, the existence of *N. ceranae* has been confirmed in the America and Asia (Chauzat et al. 2007, Cox-Foster et al. 2007, Klee et al. 2007, Huang et al. 2007, Paxton et al. 2007, Chen et al. 2008, Sarlo et al. 2008, Williams et al. 2008). Scientists has also been suggested that *N. ceranae* may replace with *N. apis* at the same time period (Klee et al. 2007, Martin-Hernandez et al. 2007, Paxton et al. 2007, Higes et al. 2009, Yoshiyama and Kimura 2011). It has been reported by scientists in many countries that *N. ceranae* has spread all over the world. (Klee et al. 2007, Fries 2010, Higes et al. 2010, Ivgin Tunca et al. 2016, Mohammadian et al. 2019, Shumkova et al. 2020). It was reported that *N. apis* cause infection in the middle intestinal epithelium of adult bees (Fries et al. 2006, Huang et al. 2007), while *N. ceranae* infects other tissues and impairs intestinal tissue integrity (Chen et al. 2009, Gisder et al. 2010, Dussaubat et al. 2012).

Nosema spores depend on their hosts to meet the ATP requirement and use transporters to draw energy from the host cell (Paldi et al. 2010). It has been shown that the routine regeneration in the intestines is not possible because of the suppression of the genes that sustain homeostasis in colonies infected with *N. ceranae* and early death occurs (Dussaubat et al. 2012). Latest, Higes et. al. (2020) examined tissue tropism of *N. apis* and *N. ceranae* in worker honey bees as well. It has been shown that the expression of the gene encoding vitellogenin (Vg), a glycolipoprotein produced and stored in the honey bees' fat body, is significantly reduced in bees infected with *N. ceranae* (Antunez et al. 2009, Goblirsch et al. 2013, Garrido et al. 2016, Badaoui et al. 2017). Recent studies have shown that *N. ceranae* C-type nosemosis has been reported to be the most common bee pathogen and has a major impact on global colony losses (Higes et al. 2007, 2010, 2013, Paxton et al. 2007, Cox-Foster et al. 2007, Fries 2010).

The first detection of Nosema disease in Turkey were reported in 1952 (Uygur and Girişgin 2008) and the presence and effects of Nosema disease were first identified by Kutlu (1988) in the Eastern Mediterranean (Adana) and the southwestern Aegean (Muğla). In the following years, molecular studies were carried out the existence *of N. apis and N. ceranae* by 3 different researchers in the same year. (Utuk et al. 2010, Muz et al. 2010, Whitaker et al. 2010). Later studies, *N. ceranae* has shown to be more common in our country (Ivgin Tunca et al. 2016, Sarıbıyık and Özkırım 2018).

In 2020, Tokarev et al (2020) informed that “the family Nosematidae is redefined and includes the genera Nosema and Vairimorpha comprising a monophyletic lineage of Microsporidia” However, Grupe and Quandt, (2020) also informed this new classification but they used as Nosema in their article in order to evaluate their study with previous literatures. In the current study, it is mentioned as Nosema in order to make a healthy comparison with the existing literatures.

The aim of this study is to determine the presence of Nosema in Muğla province in South West Anatolia. According to Beekeeping Registration System (ACS), there are 80.675 registered beekeepers and bee hives 8.12836 million in 2019 inTurkey. The registered local bee colonies in Muğla are around 1.2 million. During the pine honey production season, the number of bee hives reaches 3-3.5 million colonies with migratory beekeepers from other provinces. 90% of Turkey's pine honey is produced in Muğla. Therefore, Muğla is a very crowded and

important area for beekeeping activities. Microscopic and molecular diagnosis of Nosema spores were done from worker bee samples collected in different apiaries in 13 districts of Muğla and water samples taken from water sources which were close to apiaries. Thus, the presence and condition of Nosemosis in Muğla, which is one of the important centers for the honey bee sector, was determined by this study.

MATERIAL AND METHODS

Honey bee samples were collected from 152 different apiaries located in 13 districts of Muğla province in the spring and autumn period separately from the local and migratory beekeeping. An average of twenty hives were determined randomly from each apiary. Average five bees were taken from each hive and a total of one hundred bee samples were put into alcoholic tubes from the entrance of these selected hives from in each apiary (Table 1). At least 50 ml of water samples were taken from the 62 water sources (12 of them stable water resources inside the apiaries, rest of them water channels, streams and fountains) used by the bees near the apiaries (Table 2). Field studies were completed in May, April (Spring period) and in October-November (Autumn period) in 2017. Homogenates from honey bee samples were prepared according to the World Organization for Animal Health (OIE) application manual (2008). Detection, counting and calculation of the spores were done with thoma lame under 400x magnified light microscope using OIE terrestrial manual (2008).

Kruskal-Wallis test (SPSS 24IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) and ANOM analysis (MINITAB 18) were applied in order to determine the differences between sampling locations according to spor numbers.

DNA isolations were made in samples having highest number of spores taken from an average of 3 apiaries with from 13 districts. Commercial isolation kit (PureLinkGenomic DNA Mini Kit) was used for the isolation study. 218MITOC For-Rev and 321APIS For-Rev primers were used replicate the relevant gene regions.

Each PCR reaction mixture contained 10 ng DNA, 0.5 U Taq DNA polymerase, 10XPCR buffer, 2.5 M $MgCl_2$, 0.4 µM 218MITOC F/R, 0.5 µM 321APIS F/R primers and deonized water. Final concentration volume was 20 µl. PCR conditions included initial denaturation at 95°C for 2 min and 35 cycles were performed in 45 seconds at 95°C, 45 seconds at 59.3°C, and 1 minute at 72°C and finally 7 minutes at 72°C. PCR products were controlled in 2% agarose gel (Fries et al. 2013, Martín-Hernández et al. 2007). A total of 35 PCR products were sent to sequencing but 30 of them gave evaluating results. 30 sequence results were analyzed using MEGA 6.0 (Tamura et al. 2013), phylogenetic tree image obtained in SplitsTree (Huson and Bryant, 2006), and Network (Bandelt et al. 1999), haplotype determination was made using the program and sequence similarity analysis was performed via BLAST database.

RESULTS

The different densities of the spores were found in almost all of the samples from different apiaries (Figure 1).

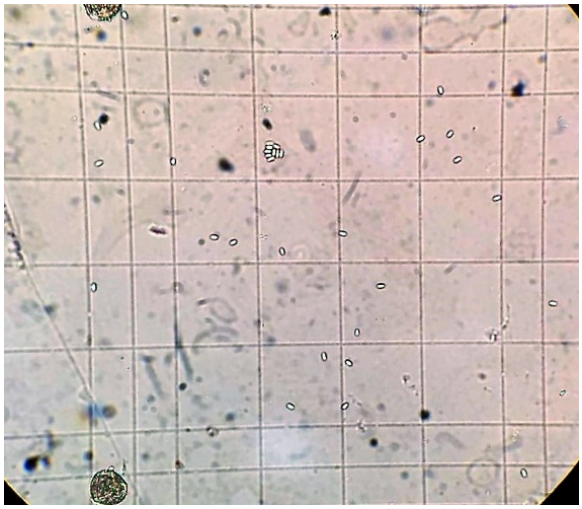

Figure 1. Nosema spores from bee samples at 400x microscope

In general, Nosema spores were found in all samples (100%) from Dalaman. This was followed by Yatağan with 92%, Milas with 90%, Ula with 84%, Ortaca with 83%, Marmaris with 80%, Köyceğiz with 75%, Bodrum with 70%, Menteşe with 69%, Fethiye with 54%, Seydikemer with 30% and Kavaklıdere with 29% (Table 1). As a result, Nosema spores caused Nosema disease by 71.53±6.02% were found throughout Muğla. The highest number of spores was observed from Yatağan samples and the lowest one was observed for samples from

Seydikemer honeybee samples (Table 1). A total of 62 water samples were examined and an average of 5.16 ± 0.64 water samples were analyzed at each location. Nosema spores were not found in the water samples taken from Menteşe, Marmaris, Datça, Bodrum, Seydikemer, Ortaca, Köyceğiz and Fethiye (Table 1). However, the spores were determined in one sample from Kavaklıdere, Dalaman and Yatağan, and in 2 samples taken from Milas and there were no water samples from Ula.

Table 1. The number of apiaries where bee samples were collected, Nosema positive apiary number, the rate of the positive apiaries, and Nosema spore numbers, the number of water samples, spore positive water samples, and ratio of positives.

Location	Number of sampled Apiaries	Number of spore positive apiaries	Ratio(%)	Spore numbers	Numbers of water samples	Positive water	Ratio(%)
Menteşe	13	9	69.23	$2.1X10^6$	3	0	0%
Marmaris	10	8	80.00	$0.7 X10^6$	4	0	0%
Milas	10	9	90.00	$0.8X10^6$	7	2	28.57%
Datça	12	7	58.33	$1X10^6$	7	0	0%
Dalaman	13	13	100.00	$2.2X10^6$	5	1	20%
Bodrum	10	7	70.00	$1X10^6$	8	0	0%
Seydikemer	13	4	30.77	$0.3X10^6$	9	0	0%
Ortaca	6	5	83.33	$1.9X10^6$	6	0	0%
Köyceğiz	4	3	75.00	$1.3X10^6$	2	0	0%
Ula	19	16	84.21	$1X10^6$	No sample		
Fethiye	22	15	68.18	$0.5X10^6$	3	0	0%
Yatağan	13	12	92.31	$9.3X10^6$	5	1	20%
Kavaklıdere	7	2	28.57	$2.6X10^6$	3	1	33.33%

Normal distribution tests (Kolmogorov-Smirnov and Shapiro-Wilk) were applied to decide which tests can be performed before starting the statistical analysis of the data obtained from the spore numbers. When all data were evaluated together, it was determined that the data did not show normal distribution ($P < 0.05$; 0.01). The same results were found when normal distribution tests were performed for the measurements made on the basis of the districts where samples were collected. Since the data sets do not show normal distribution, the nonparametric alternative of ANOVA, Kruskal-Wallis Test (SPSS 24) was applied. According to the result of Kruskal-Wallis analysis, there are statistical differences between districts in terms of the number of spores ($P < 0.01$) (Figure 2).

One-Way ANOM test was performed using Minitab 18 program to determine which districts differ in terms of the number of spore (Figure 3). In the graph, the number of spores obtained from Yatağan location (red box) is higher than other districts and have created a statistically significant difference.

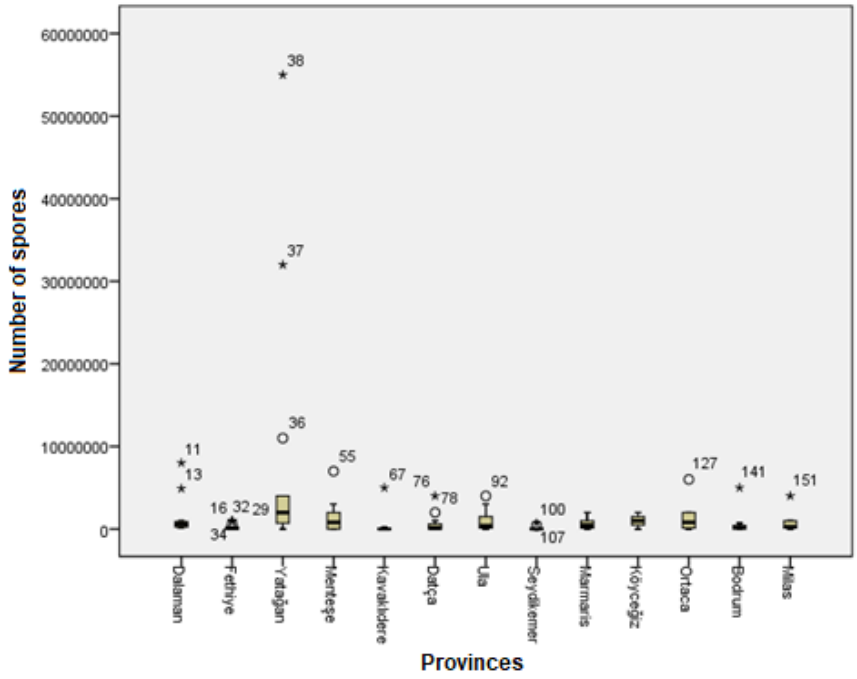

Figure 2: Box-plot for the distribution of spores' value in the samples collected areas. Circle and asterix indicated samples having extreme number of spores. The numbers above the circles and asterix are the sample order. The vertical axis shows the spore numbers and the names of locations where samples were collected on the horizontal axis are given.

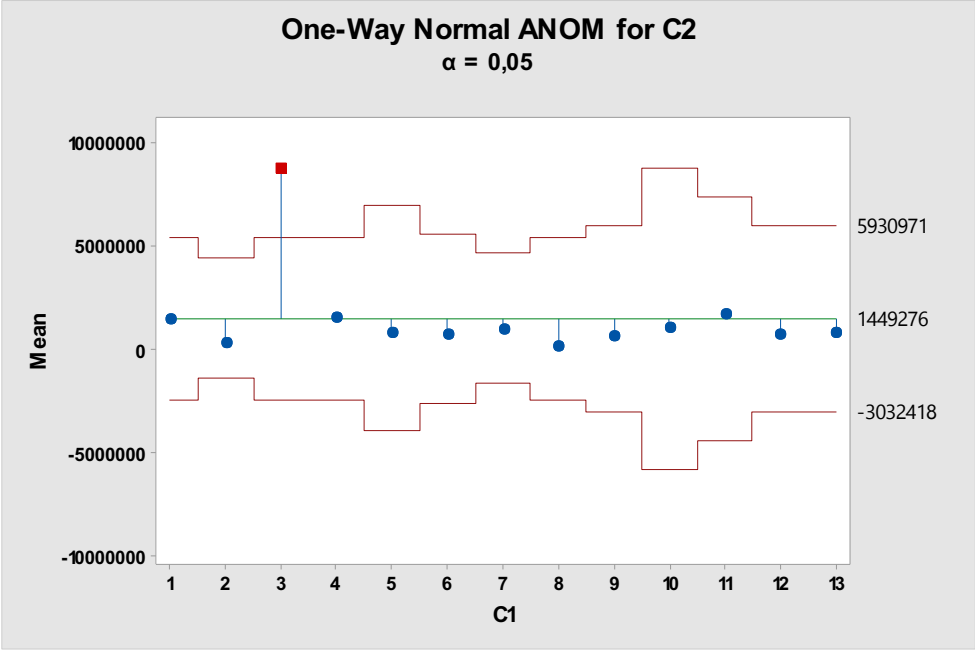

Figure 3: One-Way ANOM Test (1: Dalaman, 2: Fethiye, 3: Yatağan, 4: Menteşe, 5: Kavaklıdere, 6: Datça, 7: Ula, 8: Seydikemer, 9: Marmaris, 10: Köyceğiz, 11: Ortaca, 12: Bodrum, 13: Milas) *Red lines indicate upper and lower boundaries. The blue dots indicate the locations that do not differ, and the red box indicates the location that differs from the general average in terms of the number of spores.*

Molecular Analysis Results

Molecular analysis has shown that the observed spores belong to *N. ceranae* and *N. apis* spores were not found in this study. The sequence result of only 30 of the 35 samples, which sent to the DNA sequence, could be evaluated. As a result of the BLAST analysis, the samples showed 94-100% similarity with the *Nosema ceranae* sequences determined in Lebanon, France, Thailand and Morocco samples.

Hit sequence sequences were aligned on the MEGA 6 (Tamura et al. 2013) program. As a result of the Network (Bandelt et al. 1999) analysis, it was determined that all samples formed by 3 haplotypes according to studied sequences for the first time in Turkey. Haplotype analysis was performed to determine common genomic sequences shared by all individuals in the studied populations. The widest frequency was obtained in haplotype 1. The out group was in haplotype 1 and the out group referans sequence was from NCBI gene data bank (GenBank: Accession LC510254.1). According to the data obtained from the sequence results, haplotype 1 was detected from 21 sequenced samples from 13 location in Muğla and one sequence data from out group. Haplotype 2 was detected only in 8 samples belonging to Datça, Yatağan, Ula, Ortaca, Milas and Menteşe locations. Haplotype 3 was detected in only one sample belonging to the Ula location (Figure 4). A philogram constracted in SplitsTree (Huson and Bryant, 2006) was drawn to visualize the genetic similarities or differences identified in the populations studied (Figure 4). According to the phylogenetic tree, the Ula and out group (GenBank: Accession LC510254.1) were in different branch, the other groups from Muğla were located in other main branch.

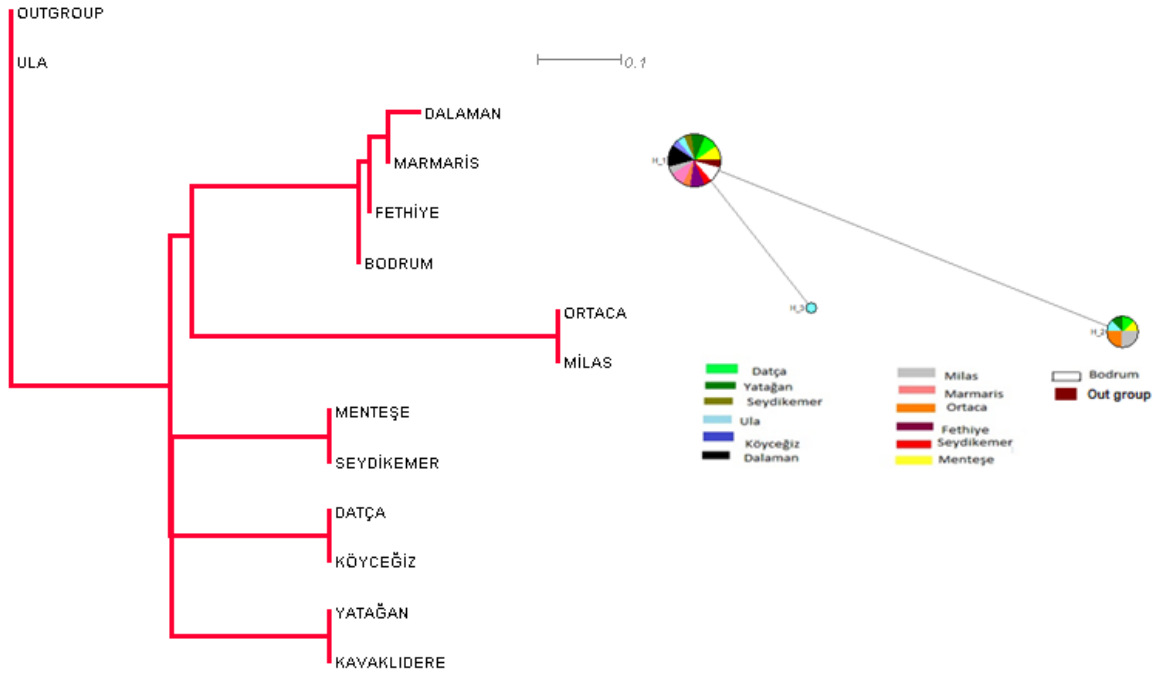

Figure 4: Dendogram from sequenced data for *N. ceranae* and the haplotypes obtained as a result of Network analysis of genes belonging to *N. ceranae*

DISCUSSION

Molecular detection of the animal and human pathogens is known to be more sensitive than microscopic analysis (Fayer et al. 2003, Giersch et al. 2009, Kahler and Thurston-Enriquez 2007, Valencakova et al. 2011). The most reliable way to distinguish and diagnose the *N. ceranae and N. apis* is using molecular methods (Gatehouse and Malone 1998, Sagastume et al. 2010, Tay et al. 2005). Nosema disease is effective all over the world as well as in our country. From time-to-time Nosematosis cause significant colony losses. In our country, the first findings of Nosema disease were reported in 1952 and other studies on Nosema disease continued in later years (Uygur and Girişgin 2008, Büyük et al. 2014). The prensent study, Nosema spores detected from worker honeybees and water sources found in or near the apiaries. Statistical differences between Nosema spores were found to be significant among the sampled locations in Muğla. In the study, it is thought that number of Nosema spores increase in the hive due to heavy rainfall and humidity in the spring. Previous studies have also reported that the linear relationship between Nosema spore density and humidity is statistically significant (Büyük 2016, Tosun 2012, Gisder et al. 2010, Martín-Hernández et al. 2009). Traver and Fell (2012,) also mentioned that *N. ceranae* spores have been reported to occur at high levels in spring and low levels in fall and winter. In the study, a very small proportion of the collected water samples were contaminated by nosema spores according to the water analysis. Water samples were collected from apiaries (the bee samples were taken in the same apiaries) or near the apiaries. The few number of spores were found in the water samples due to the fact that the water sources were mostly them are flowing water (stream, water channel, fountain, etc.). But nematodes and protozoa species have been observed rather than the spores. In addition, the pollution was observed in the water containers placed in the apiaries in order to meet the water supply fro the bees. Because the water containers were not cleaned and changed frequently enough. At this point, it should be taken into account that bees benefiting from stable water resources may be exposed to other diseases due to dirty containers and water. In the current study, *N. apis* were not found both molecular and microscopic analyses in worker bees and water samples. *N. ceranea* spores were the only spores in both sample types.

The distribution and effects of Nosema disease in Adana and Muğla provinces was carried out by Kutlu (1988) and 15600 worker honey bee samples collected from 312 apiaries were studied as a result of microscopic analyses. Their results showed that the disease level was determined as 31.3% in Muğla, 29.8% in Adana, 29.6% in Dalaman, 28.6% in Aydın, 25.7% in Datça, 25.0% in Milas, 23.8% in Fethiye, 23.3% in Köyceğiz and 20.5% in Marmaris. In present study, the ratio of disease in all Mugla region is 71.53%. A 2.5-fold increase in the percentage of disease is observed from Kutlu's study in 1988 to 2017 in which our study was conducted. *N. ceranae* has a more severe effect than *N. apis*. The study was conducted in 1988 on the basis of *Nosema apis*, whereas today *N. ceranae* has an impact on the whole region. This situation shows that the effects of Nosema disease in Muğla region are more serious.

In other study for Muğla region, Nosema was effective in winter and spring periods, and also the disease was the most intense in the Thrace region and Muğla (Başar 1990). Another study investigated the density of *N. apis* on 7820 honeybees between in August 1988-June 1989, they found that Nosema infection pevalance was highest in April-November (Keskin et al. 1996). At different time periods, Nosema spores were determined by microscopic method in Muğla (Şimşek 2007, Şimşek et al. 2010). According to different studies, *N. ceranae* was found from the bee samples in Muğla (Whitaker et al.,2010; Utuk et al. 2016; Ivgin Tunca et al. 2016) Sarıbıyık and Özkırım (2018) collected 51 samples from Muğla province in 2 periods including spring and August in Muğla province. In 102, they found *N. ceranae* in 20, *N. apis* in 13, and both spores in 69 samples.

Since molecular techniques were not so widespread in the past, Nosema disease was shown as *N. apis*. On the other hand, *N. ceranae* was thought to infect only *Apis ceranae* until twenty years ago. The later studies revealed that *N. ceranae* also infects western European honey bees. In a recent study in Thailand to understand the biology of *N. ceranae*, the genetic diversity in different hosts (*A. mellifera, A. ceranae*) was investigated using both PCR and genome-based methods, and that *N. ceranae* populations shared many SNPs with other global populations and it was observed to be clonal. However, on the contrary of previous studies, it has been determined that these populations carry many SNPs that are not found elsewhere, and these

populations have evolved in their current geographic location for some time (Peters et. al. 2019).

In current study, Nosema spores have not been detected in flowing water resources as a result of water analysis but it does not mean that there are no spores. This study shows that *N. ceranae* is widespread in Muğla province. At the same time, the molecular haplotype of *N. ceranae* gene regions from mediterrenean samples were determined for the first time in Turkey. It will be possible to examine the determining role of haplotypes on the wintering ability and reproductive performance of bees with current data.

As a result, beekeeping contributes the economy of the countries directly and indirectly. Bee diseases play effective role in the quality of bee products and the sustainability of colonies. Therefore, periodic monitoring of bee diseases and investigation of their effects is important in terms of sustainable beekeeping activity.

Contribution of authors as; Serengül Kartal, Rahşan İvgin Tunca, Hasan Koç, Okan Özgül for sample collection, Serengül Kartal, Rahşan İvgin Tunca for lab. analysis, Serengül Kartal, Kemal Karabağ for statistical anlysis, Serengül Kartal, Rahşan İvgin Tunca, Kemal Karabağ, Hasan Koç, Okan Özgül for ms writing.

Conflict of interest: The *authors* declare that *here is no conflict of interest* regarding the publication of this article

Financial Aid: The study was supported by Muğla Sıtkı Koçman University Scientific Research Projects Coordinator with project number 17/010.

Acknowledgement

The *authors* declare that *there is no conflict of interest* regarding the publication of this article. In addition, we would like to thank Muğla Provincial Directorate of Agriculture, Muğla Bee Keeping Association who provided to supply of the samples, MSKU Scientific Research Projects Coordinator and Research Assistant Emel TÜTEN SEVİM for helps. This article was created from Serengül KARTAL's Master Thesis.

REFERENCES

Antúnez, K., Martin-Hernandez, R., Prieto, L., Meana, A., Zunino, P., Higes, M., (2009). Immune Suppression in the Honey Bee (*Apis mellifera*) Following Infection by *Nosema ceranae* (Microsporidia), Environ. Microbiol., doi:10.1111/j.1462- 2920.2009.01953x.

Badaoui F., Amar A., Hassou L., Zoglat A., Okou CG. (2017). Dimensionality Reduction And Class Prediction Algorithm With Application To Microarray Big Data, Journal of Big Data, 4: 32. https://doi.org/10.1186/s40537-017-0093-4.

Bandelt, HJ., Forster, P., Röhl, A., (1999). Median-Joining Networks For Inferring Intraspecific Phylogenies, Molecular Biology and Evolution, 16:37-48.

Başar, E. (1990). Ülkemizdeki Bal Arılarında *(Apis mellifera) Acarapis woodi* ve *Nosema apis* Parazitlerinin Araştırılması, Yüksek Lisans Tezi, Hacettepe Üniversitesi, Fen Bilimleri Enstitüsü.

Büyük, M., (2016). Kırşehir İlindeki Arılarda Nosema Hastalığının Belirlenmesi, Yüksek lisans Tezi Kırşehir Ahi Evran Üniversitesi, Fen Bilimleri Enstitüsü, Zootekni Anabilim Dalı.

Büyük, M., Tunca, Rİ., Taşkın, A. (2014). 'Türkiye'de Nosema spp. Varlığına Yönelik Yapılmış Çalışmalar', Türk Tarım ve Doğa Bilimleri Dergisi, 1(2), 234–238.

Chauzat, MP., Higes, M., Martín-Hernández, R., Meana, A., Cougoule, N., Faucon, JP. (2007). Presence of *Nosema ceranae* in French Honey Bee Colonies, J. Apic. Res. 46, 127–128.

Chen, Y., Evans, JD., Smith, IB., Pettis, JS. (2008). *Nosema ceranae* is a Long-Present And Wide-Spread Microsporidian Infection of the European Honey Bee (*Apis mellifera*) in the United States, J. Invertebr. Pathol. 97, 186–188.

Chen YP, Evans JD, Murphy C, Gutell R, Zuker M, Gundensen- Rindal D, Pettis JS (2009). Morphological, Molecular, And Phylogenetic Characterization of *Nosema ceranae*, a Microsporidian Parasite Isolated From The European Honey Bee, *Apis mellifera*, J Eukaryot Microbiol 56:142–147.

Cox-Foster, DL., Conlan, S., Holmes, EC., Palacios, G., Evans, JD., Moran, NA., Lipkin, WI.

(2007). A Metagenomic Survey of Microbes in Honey Bee Colony Collapse Disorder, Science, 318, 283–287.

Doğaroğlu, M. (1999). Modern Arıcılık Teknikleri, Anadolu Matbaa ve Ambalaj San. Tic. Ltd. Sti., İstanbul.

Dussaubat, C., Brunet, JL., Higes, M., Colbourne, JK., Lopez, J., Choi, JH., Martín-Hernández, R., Botias, C., Moritz, RF., Le Conte, Y., Alaux, C, (2012). Gut Pathology And Responses To The Microsporidium, *Nosema ceranae* in the Honey Bee Apis mellifera, PLoS One, 7(5): e37017.

Fayer, R., Santín, M., Trout, JM., (2003). First Detection Of Microsporidia in Dairy Calves in North America, Parasitol Res 90:383–386.

Fries, I. (2010). *Nosema ceranae* in European Honey Bees, Journal of Invertebrate Pathology 103, 73-79.

Fries, I., Chauzat, MP., Chen, YP., Doublet, V., Genersch, E., Gisder, S., Higes, M., Mcmahon, DP., Martínhernández, R., Natsopoulou, M., Paxton, RJ., Tanner, G., Webster, TC., Williams, GR. (2013). Standard Methods for *Nosema* research. *In:* The COLOSS BEEBOOK: Volume II: Standard methods for *Apis mellifera* Pest and Pathogen Research, Dietemann V, Ellis J.D. & Neumann P, eds. Journal of Apicultural Research, 52 (1):http://dx.doi.org/10.3896/IBRA.1.52.1.14

Fries, I., Feng. F., Da Silva, A., Slemenda, SB., Pieniazek, NJ. (1996). *Nosema ceranae* (Microspora, Nosematidae), Morphological And Molecular Characterization Of A Microsporidian Parasite Of The Asian Honeybee *Apis cerana* (Hymenoptera, Apidae), European Journal of Protistology 32, 356-365.

Fries, I., Martín, R., Meana, A., García-Palencia, P., Higes, M. (2006). Natural Infections of *Nosema ceranae* in European Honeybees, Journal of Apicultural Research 45, 230-233.

Garrido, PM., Porrini, MP., Antúnez, K., Branchiccela, B., Martinez-Noel, GMA., Zunino, P., Salerno, G., Eguaras, MJ., Leno, E., (2016). Sublethal Effects Of Acaricides and *Nosema ceranae* Infection On Immune Related Gene Expression in honeybees, Garrido *et al. Vet Res 47:51*DOI 10.1186/s13567-016-0335-z

Gatehouse, HS., Malone LA., (1998). The Ribosomal RNA Gene Region of *Nosema apis* (Microspora): DNA Sequence for Small and Large Subunit rRNA Genes and Evidence of a Large Tandem Repeat Unit Size, J Invertebr Pathol. Mar;71(2):97-105.

GenBank: ACCESSION No LC510254.1 AUTHORS Takashima, S., Ohari, Y. and Itagaki, T. TITLE The prevalence of newly found Nosema species and Nosema ceranae from honeybees, Apis cerana japonica and Apis mellifera in Tohoku region of Japan.

Giersch, T., Berg, T., Galea, F., Hornitzky, M. (2009). *Nosema ceranae* Infects Honeybees (*Apis mellifera*) and Contaminates Honey in Australia, Apidologie 40, 117-123.

Gisder, S., Hedtke, K., Möckel, N., Frielitz, MC., Linde, A., Genersch, E. (2010). Five Year Cohort Study Of *Nosema* spp. in Germany: Does Climates Hape Virulence And As Sertiveness Of *Nosema ceranae*?, *Applied* and *Environmental Microbiology*76**,**3032–3038.

Grupe, A.C. II., Quandt, C.A. (2020) A growing pandemic: A review of *Nosema* parasites in globally distributed domesticated and native bees. PLOS Pathogens 16(6): e1008580. https://doi.org/10.1371/journal.ppat.1008580

Goblirsch, M., Huang, ZY., Spivak, M., (2013). Physiological and Behavioral Changes in Honey Bees (*Apis mellifera*) Induced by *Nosema ceranae* Infection, https://doi.org/10.1371/journal.pone.0058165

Gülpınar, V. (2005). Bal Arısı Hastalık ve Zararlıları, *Teknik Arıcılık*. 87: 2-7.

Higes M, Garcia-Palencia P, Martín-Hernández R, Aranzazu M. (2007). Experimental Infection of *Apis mellifera* Honey Bees with *Nosema ceranae* (Microsporidia), J Invertebr Pathol, 94(3): 211-217.

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., González-Porto, AV., García-Palencia, P., Meana, A., et al. (2009). Honeybee Colony Collapse due to *Nosema ceranae* in Professional Apiaries, Environmental Microbiology Reports1,110–113.

Higes, M., Martín-Hernández, R., Meana, A. (2006). *Nosema ceranae,* A New Microsporidian Parasite in HoneyBees in Europe, Journal of Invertebrate Pathology 92, 93–95.

Higes, M., Martín-Hernández, R., Meana, A. (2010), *Nosema ceranae* in Europe: An Emergent Type C nosemosis, Apidologie 41,375–392.

Higes, M., Meana, A., Bartolomé, C., Botías, C., Martín-Hernández, R. (2013). *Nosema ceranae* (Microsporida), a Controversial 21st Century Honeybee Pathogen, Environmental Microbiology Reports 5,17–29. doi:10.1111/1758-2229.12024.

Mariano Higes, Pilar García-Palencia, Almudena Urbieta, Antonio Nanetti, Raquel Martín-Hernández (2020). *Nosema apis* and *Nosema ceranae* Tissue Tropism in Worker Honey Bees (*Apis mellifera*) Veterinary Pathology 57(1) 132-138. https://doi.org/10.1177/0300985819864302

Huang, WF., Jiang, JH., Chen, YW., Wang, CH. (2007). *A Nosema ceranae* Isolate from The Honeybees *Apis mellifera*, Apidologie 38, 30-37.

Huson, DH., Bryant H., (2006) Application of Phylogenetic Networks in Evolutionary Studies, Mol. Biol. Evol., 23(2):254-267.

İvgin Tunca, R., Oskay, D., Gösterit, A., Tekin, K., (2016). Does *Nosema ceranae* Wipe Out Nosema apis in Turkey? (Short Communication), Iranian Journal of Parasitology, 11(2):259-264.

Kahler, AM., Thurston-Enriquez, JA., (2007). Human Pathogenic Microsporidia Detection in Agricultural Samples: Method Development And Assessment, Parasitol Res 100:529–538.

Keskin, N., Basar, E., Saraçbaşı, T., (1996). Türkiye'nin Bazı Yörelerindeki Bal Arılarında (*Apis mellifera* L.) Nosema Hastalığı, Hacettepe Fen ve Mühendislik Bilimleri Dergisi, 17, 25-35.

Klee J, Besana A, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. (2007). Wide Spread Dispersal Of The Microsporidium *Nosema ceranae*, An Emergent Pathogen Of The Western Honeybee, *Apis mellifera*, Journal of Invertebrate Pathology, 96,1–10.

Kutlu, MA. (1988). Ergin Balarısı (*Apis mellifera* L.) Hastalığı *Nosema apis*'in Dağılımı ve Enfeksiyon Oranı Üzerine Bir Araştırma, Yüksek Lisans Tezi, Çukurova Üniversitesi, Fen Bilimleri Enstitüsü, Adana, 45 s.

Martín-Hernández, R., Meana, A., Garcia-Palencia, P., Marin, P., Botias, C., Garrido-Bailon, E., *et al.* (2009). Effect of Temperature on The Biotic Potential of Honeybee Microsporidia, Applied and Environmental Microbiology, 75, 2554–2557.

Martín-Hernández, R., Meana, A., Prieto, L., Martinez- Salvador, A., Garrido- Bailon, E., Higez, M. (2007). Outcome of Colonization of *Apis mellifera* by *Nosema ceranae*, Applied and Environmental Microbiology, 73, 6331-6338.

Matheson, A. (1996). World Bee Health Update, Bee World, 77: 45–51.

Mohammadian, B., Bokaie, S., Moharrami, M., Nabian, S., Forsi, M. (2019). Prevalence of Honeybee Colony Collapse Disorder and İts Relation To *Nosema* spp. and Climate In Apiaries Of Ira', Journal of Veterinary Research, 74(1):11-18 10.22059/JVR.2017.235690.2649

Muz, MN., Girisgin, AO., Muz, D., Aydin, L. (2010). Molecular Detection Of *Nosema ceranae* and *Nosema apis* Infections in Turkish Apiaries With Collapsed Colonies, Journal of Apicultural Research, Vol. 49 **(**4), 342.

Özbek, H., (2002). Arılar ve Doğa, Uludağ Arıcılık Dergisi, 2 (3): 22-25.

Öztürk, Aİ. (2001). Balarısı Hastalıkları, Muğla'da Tarım, 1(5): 57-59.

Paldi N., Glick, E., Oliva, M., Zilberberg, Y., Aubin, L., Pettis, J., Chen, Y., Evans, JD., (2010). Effective Gene Silencing in a Microsporidian Parasite Associated with Honeybee (*Apis mellifera*) Colony Declines, Appied and Environmental Microbiology, 76(17): 5960–5964.

Paxton, RJ. (2010). Does Infection by *Nosema ceranae* Cause "Colony Collapse Disorder" in Honeybees (*Apis mellifera***),** Journal of Apicultural Research 49, 80-84.

Paxton, RJ., Klee, J., Korpela, S., Fries, I. (2007). *Nosema ceranae* Has Infected *Apis mellifera* in Europe Since At Least 1998 and May Be More Virulent Than *Nosema apis.* Apidologie, 38,558-565.

Peters, M.J, Suwannapong, G., Pelin, A., Corradi, N., (2019). Genetic and Genome Analyses Reveal Genetically Distinct Populations of the Bee Pathogen *Nosema ceranae* from Thailand. Microbial Ecology 77(4):877-889. doi: 10.1007/s00248-018-1268-z.

Sagastume, S., del Åguila, C., Martín-Hernández, R., Higes, M., Henriques-Gil, N., (2010). Polymorphism And Recombination For rDNA In The Putatively Asexual Microsporidian *Nosema ceranae*, A Pathogen Of Honey Bees, Environmental Microbiology, 13(1): 84-95. http://dx.doi.org/10.1111/j.1462-2920.2010.02311.x.

Sarıbıyık, C., Özkırım, A. (2018), The Transition Ratio of *Nosema* spp. Spores From Colonies to Honey Versus Honey to Colonies, Journal of Agricultural Science; Vol. 11, No. 1; 2019, 72-80.

Sarlo, E., Medici, SK., Braunstein, M., Eguaras, M., (2008). Presencia Y Distribución De *Nosema ceranae* En La Región Sudeste De La Provincia De Buenos Aires, In: Actas del Segundo Congreso Argentino de Apicultura, Mar del Plata, Argentina, Agosto 2008, p. 26.

Shumkova, R., Neov, BS., Georgieva, A., Teofanova, D., Radoslavov, G., Hristov, P. (2020). Resistance of Native Honey Bees from Rhodope Mountaıns and Lowland Regions of Bulgarıa to *Nosema ceranae* and Viral Pathogens, Bulgarian Journal of Veterinary Medicine, 2020, 23, No 2, 206–217 DOI: 10.15547/bjvm.2201.

Şimşek D., (2007). Muğla İli Balarılarının (*Apis mellifera* L.) Mikrobiyal ve Paraziter Hastalıklar Yönünden İncelenmesi, Hacettepe Üniversitesi Fen Bilimleri Entitüsü, Yüksek Lisans Tezi, Ankara.

Şimşek, D., Keskin, N., Aktaş, S., (2009) Türkiye, Arıcılık Endüstrisinde Önemli Bir Yere Sahip Olan Muğla'da Nosemosis Üzerine Bir Araştırma, Mellifera, 9, 2-8.7.

Tamura, K., Stechr, G., Peterson, D., Filipski, A., Kumar, S., (2013). MEGA 6: Molecular Evolutionay Genetics Analysis Version 6.0. Molecular Biology and Evolution: 30 2725-2729.

Tay, WT., O'mahoney, EM., Paxton, RJ., (2005). Complete rRNA Gene Sequences Reveal That The Microsporidium *Nosema bombi* Infects Diverse Bumble Bee (*Bombus* spp.) Hosts and Contains Multiple Polymorphic Sites, Journal of Eukaryotic Microbiology, 52(6): 505-513. http://dx.doi.org/10.1111/j.550-7408.2005.00057.x.

Tokarev, Y.S., Huang, W. F., Solter, L.F., Malysh, J. M., Becnel, J. J., Vossbrinck, C. R., (2020) A formal redefinition of the genera Nosema and Vairimorpha (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics, Journal of Invertebrate Pathology 169: 107279. doi.org/10.1016/j.jip.2019.107279

Tosun, O. (2012). Bal Arılarında (*Apis mellifera* L., 1758) Nosemosis (Nosematosis) Hastalığının Doğu Karadeniz Bölgesinde Bulunan Arı Kolonilerindeki Varlığı, Dağılımı ve Hastalık Etkenlerinin Karakterizasyonu, Doktora Tezi, Karadeniz Teknik Üniversitesi, Fen Bilimleri Enstitüsü, Biyoloji Anabilim Dalı, Trabzon, 112 s.

Traver, BE., Fell, RD. (2012). Prevalence and Infection Intensity of Nosema in Honey Bee (Apis mellifera L.) Colonies In Virginia, J. Invertebr. Pathol., 107, 43–49.

Uygur, ŞÖ., Girişgin, AO. (2008). Bal Arısı Hastalık ve Zararlıları, Uludağ Arıcılık Dergisi, 8, 4,130-142.

Ütük, A.E., Pişkin, F.Ç., Kurt, M. 2010. Türkiye'de Nosema ceranae'nın ilk moleküler tanısı. Ankara Üniversitesi Veterinerlik Fakültesi Dergisi, 57: 275-278.

Ütük, AE., Piskin, FÇ., Girişgin, AO., Özgür, S., Aydın, L., (2016). Microscopic and Moleculer Detection of *Nosema* spp. In Honeybees of Turkey, Apidologie 47: 267-271.

Van Engelsdorp, D., Meixner, MD., (2010). A Historical Review of Managed Honey Bee Populations in Europe and the United States and the Factors that May Affect Them, Journal of Invertebrate Pathology, 580-595.

Valencakova, A., Balent, P., Ravaszova, P., Horak, A., Obornik, M., Halanova, M., Malcekova, B., Novotny, F., Goldova, M., (2011). Molecular Identification and Genotyping Of Microsporidia in Selected Hosts, Parasitol Res. doi:10.1007/s00436-011-2543-9.

Whitaker, J., Szalanski, AL., Kence, M. (2010). Molecular Detection of *Nosema ceranae* and *N. apis* from Turkish Honey Bees, Apidologie doi:10.1051/apido/2010045.

Williams, GR., Shafer, ABA., Rogers, REL., Shutler, D., Stewart, DT. (2008). First detection of *Nosema ceranae*, a Microsporidian Parasite of European Honeybees (*Apismellifera*), in

Canada and Central USA., Journal of Invertebrate Pathololology, 97,189–192.

Yoshiyama, M., Kimura, K. (2011). Distribution of *Nosema ceranae* in the European Honey Bee, *Apis mellifera* in Japan, Journal of Invertebrate Pathology, 106, 263–267.

Zander, E. (1909). Tierische Parasiten als Krankenheitserreger bei der Biene. Münchener Bienenztg, 31, 196–204.