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ORIGINAL PAPER

PCR-Based Screening of Pathogens in *Bombus terrestris* Populations of Turkey

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Abstract

Purpose Bumblebees are an important group of insects in the pollination of various vegetables, fruits, oilseeds, legumes, and the fodder crops. Compared to honeybees, they have a wider choice of hosts and a longer flight period. These bees are used especially for the pollination of plants in greenhouses and are commercially produced for this purpose. Recently, serious decreases have been occurring in bumblebee populations due to various reasons such as pathogens, and some of species are even threatened with extinction. Due to the worldwide decline in pollinator insects, determining the distribution and prevalence of bumblebee pathogens is of great importance. Therefore, this study was conducted to determine the incidence and prevalence of pathogens in Turkish bumblebee populations and how much of each pathogen was in bumblebee samples.

Methods A total of 172 *Bombus terrestris* (Linnaeus,1758) samples (21 samples from commercial enterprises, 79 samples from greenhouses and 72 samples from nature) were randomly collected from 3 provinces (Antalya, Mersin and İzmir) where greenhouse cultivation is intensively carried out in Turkey. Eighty-nine of these samples were collected in the spring and eighty-three in the autumn. The presence of four pathogens (*Nosema bombi*, *Crithidia bombi*, *Apicystis bombi*, and *Locustacarus buchneri*) was investigated by PCR using universal primers.

Results The overall prevalence of *Nosema bombi*, *Crithidia bombi*, *Apicystis bombi*, and *Locustacarus buchneri* was determined as 7.55%, 9.3%, 11.62%, and 4.65%, respectively. Co-infections (5.81%) were only detected in wild-caught (nature) samples. *C. bombi* and *A. bombi* infections were detected at higher rates in the spring samples than in the autumn samples ($p < 0.05$). There was no significant difference between the spring and autumn samples with respect to the presence of *N. bombi* and *L. buchneri* ($p > 0.05$).

Conclusion The results obtained could be important in determining the prevalence and spread rates of the bumblebee diseases in Turkey and to determine appropriate protection measures. The information gathered should increase our knowledge about the presence of these pathogens in Turkey and could contribute to improve apiarist's practice. More studies are needed to determine the transmission pathways of these pathogens between the populations. Also, complex pathogen interactions in bumblebee populations should be considered in the future to improve bumblebee health.

Keywords Bumblebee · Pathogen · Parasite · Prevalence · PCR

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Introduction

Bumblebees (*Bumbus* spp.) are a social group of bee species forming the structured colonies, and the genus of *Bombus* consists of a large number of species (about 260 species) that are mostly found in the northern hemisphere [1, 2]. *Bombus* species are important insects in the pollination of greenhouse crops such as tomatoes and peppers and some of the species in this genus commercially produced and used for pollination purposes. Colonies of some species such as the European *B. terrestris* (L. 1758) (Hymenoptera: Apidae) and the North American *B. impatiens* Cr. (Hymenoptera: Apidae) are transported all over the world since they are highly effective in pollination and are easy to use [2, 3]. More than 1 million bumblebees are commercially produced in the world for each year, more than 90% of them are *B. terrestris* [4]. Although bumblebees are vitally important pollinators of both natural and agricultural ecosystems, decreases have been detected in their populations due to agricultural concentration, urbanization, pesticide use, climate change, reduced flower resources, and habitat fragmentation [5, 6]. It has been shown by many studies that one of the most important reasons for these population decreases is associated with pathogens infecting bumblebees [7–10]. Nowadays, morphological methods are used to detect pathogens in both honeybees and bumblebees, but lately PCR-based molecular methods have been frequently used due to their high specificity, sensitivity, more accurate and reliable results [6, 11–14].

Colonies of naturally and commercially produced bumblebees are attacked by many pathogens, and as a result, bees can die and sometimes colony deaths can occur. The most common of these pathogens are the microsporodium *Nosema bombi*, the protists *Crithidia bombi* and *Apicystis bombi*, the tracheal mite, *Locustacarus buchneri* Stammer 1951 (Acari; Podapolipidae), and the nematode *Sphaerularia bombi* (Nematoda: Sphaerulariidae) [6, 15]. Knowing the distribution and prevalence of pathogens which are present in natural and greenhouse bumblebee colonies is very important in elucidating the factors that cause population decline of these bees. In addition, it is certain that the data obtained will have an important point in the control of bumblebee diseases and in determining the spread rates of the diseases. For this purpose, the studies on pathogens of bumblebee colonies in Turkey are very limited. For instance, Eldeniz Cankaya and Kaftanoglu [16] detected *N. bombi* (12.96%), *C. bombi* (8.99%), *A. bombi* (6.89%), and *L. buchneri* (5.28%) infections in 578 *B. terrestris* queens collected from the Aegean and Mediterranean coastlines of Turkey. In the same study, the presence rate of various pathogens based on the provinces such as Adana, Mersin, Antalya, and Muğla in Turkey was determined (*N. bombi* (the highest rate 17.48%), *C. bombi* (the highest rate 9.76%), and *L. buchneri* (the highest rate 13.01%)). Finally, Aytekin *et al*. [17] investigated the presence of various pathogens in *B. terrestris* queens collected from Ankara province and its surroundings. As a result, they detected *Acarus farris* (Ousemans, 1905) (Acari: Acaridae) and *N. bombi* in 20.9% and 74.63% of samples, respectively.

In this study, the presence of *N. bombi*, *C. bombi*, *A. bombi*, and *L. buchneri* in a total of 172 *B. terrestris* samples collected from Antalya, Mersin, and İzmir provinces of Turkey (21 samples from commercial enterprises, 79 samples from greenhouses, and 72 samples from nature) was investigated by PCR using specific primers and the prevalence of diseases in the samples was revealed. The data obtained are thought to be important in determining the prevalence and spread rates of disease agents in the bumblebee populations of Turkey.

Materials and Methods

Collection of Samples

B. terrestris specimens were collected from a total of 3 provinces in Turkey (Antalya, Mersin and İzmir) in 2018, separately in spring (89 specimens) and autumn (83 specimens). In the selection of locations, places where greenhouse cultivation are carried out intensively were preferred. The collection of bee samples was performed randomly. The sampled bumblebees in commercial colonies are bred in the sample area (Turkey). The types of greenhouses samples are traditional (with a gable roof) type and mostly tomatoes and cucumbers are grown in them. A total of 172 specimens (21 from commercial colonies, 79 from greenhouses, and 72 from nature) were screened by PCR for the presence of *N. bombi*, *C. bombi*, *A. bombi*, and *L. buchneri*. The number of samples for each province is given in Table 1.

DNA Extraction

The collected bee samples were brought to the laboratory and stored at – 20 °C until DNA isolation was performed. The head, thorax, and abdomen of the bees were used for DNA isolation. First, the samples were crushed with liquid nitrogen using a mortar and pestle and 50–100 mg from the powdered samples was separately transferred into microcentrifuge tubes. After the samples were treated with proteinase K at 55 °C for 2 h, the DNA isolation was performed by ExiPrep Plus Tissue Genomic DNA Kit and the Bioneer Exiprep 16 Plus Genomic DNA innovation robot (Bioneer Corporation, Korea). The quantities of the DNA

Table 1 The provinces of Turkey where bumblebee samples were collected, the number of samples for each province, and the disease rate for each pathogen or parasite

Pathogen (% infection rate)	Provinces					
	Spring ($n=89$)			Autumn ($n=83$)		
	Antalya ($n=36$)	Mersin ($n=33$)	İzmir ($n=20$)	Antalya ($n=34$)	Mersin ($n=29$)	İzmir ($n=20$)
Nosema bombi	5.5%	9%	10%	8.8%	6.8%	5%
Crithidia bombi	19.4%	6%	30%	0%	3.4%	0%
Apicystis bombi	19.4%	15.1%	35%	2.9%	0%	0%
Locustacarus buchneri	5.5%	0%	15%	8,8%	0%	0%

samples were spectrophotometrically determined and stored at – 20 °C until use.

PCR and Sequencing

After DNA isolation from the bumblebee samples, PCR amplification was performed for the relevant gene region from the samples using the primers and temperature cycles specified in Table 2. The PCR reaction mixture for all amplifications was prepared to include 200 µM from each dNTP, 50 pmol from each primer, 2.5-unit *Taq*-DNA-polymerase, 5 µl 10 × *Taq*-DNA-polymerase reaction buffer, and 50 ng genomic DNA. Final volume was completed to 50 µl with ddH_2O. At the end of the PCRs, 5 µl of the products were electrophoresed at 90 V for 45 min with 1 kb DNA ladder on 1% agarose gel with 0.5 µg / ml ethidium bromide [18]. One PCR product for each pathogen were subjected to the sequence analysis. All DNA sequences were edited with BioEdit 7.09 [19] and then were blasted at NCBI GenBank to determine their similarities with known species to confirm the species identification [20]. GenBank submission numbers are provided in Table 3.

Statistical Analysis

The difference between the spring and autumn *B. terrestris* samples with respect to the presence of pathogens was determined by two-proportion *Z* test. The significance level of $\alpha = 0.05$ was used for statistical testing. The analyses were performed with Minitab 17.1.0 statistical software.

Results

After screening of 172 *B. terrestris* samples, the overall infection rate for *N. bombi*, *C. bombi*, *A. bombi*, and *L. buchneri* was determined as 7.55%, 9.3%, 11.62%, and 4.65%, respectively. The infection rates in spring samples were determined as 6.74% for *N. bombi*, 16.85% for *C. bombi*, 21.34% for *A. bombi*, and 5.61% for *L. buchneri*. For the autumn season, the infection rates were 7.22% for *N. bombi*,

Table 2 Primers used in this study and PCR conditions

Pathogens	Primers (forward and reverse)	Sequence (5′→3’)	PCR conditions	Band size (bp)	Reference
Nosema bombi	BOMBICAR (16S rRNA)	fwd GGCCCATGCATGGTT TTTGAAGATTATTAT rev CTACACTTTAAC GTAGTTATCTGCGG	95 °C for 10 min; 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s for 35 cycles, and 72 °C for 5 min for 1 cycle	101 bp	[36]
Crithidia bombi	CB-SSUrRNA-F2 CB-SSUr-RNA-B4 (18S rDNA)	fwd CTTTTGACGAAC AACTGCCCTATC rev AACCGAACGCACTAA ACCCC	95 °C for 5 min; 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min for 40 cycles and 72 °C for 3 min for 1 cycle	680 bp	[37]
Apicystis bombi	ApUF1 ApUR1 (18S rDNA)	fwd TCAATTGGAGGG CAAGTCTG rev CACGCAAAGTCCCTC TAAGAA	94 °C for 2 min; 94 °C for 30 s, 60.7 °C for 30 s, 72 °C for 45 s for 35 cycles and 72 °C for 3 min for 1 cycle	850 bp	[38]
Locustacarus buchneri	C1-J-1751 C1-N-2329 (mtDNA CO1)	fwd ggATCACC TgTAATAgCATTCCC rev ACTgTAAATATATgAT-gAgCTCA-3’	95 °C for 9 min; 94 °C for 30 s, 45 °C for 30 s, 72 °C for 30 s for 30 cycles and 72 °C for 7 min for 1 cycle	535 bp	[39]

Table 3 The percent similarity of pathogen's DNA sequences from *Bombus terrestris* samples

Pathogens	Gene	GenBank ID number	The most related species	GenBank ID number	Query coverage (%)	Identity (%)	Suggested identification
Nosema bombi	16S rRNA	OP738384	*Nosema bombi* isolate Q3	MK942710	100%	98.99%	*N. bombi*
			Nosema bombi isolate J46	MK942709	100%	98.99%	
			Nosema bombi isolate J25	MK942708	100%	98.99%	
Crithidia bombi	18S rDNA	OP738398	*Crithidia bombi* isolate AK08.053	KM980185	99%	98.51%	*C. bombi*
			Crithidia bombi isolate F5_CB-B4/F2	KU096060	99%	98.51%	
			Crithidia bombi	FN546181	99%	98.36%	
Apicystis bombi	18S rDNA	OP738417	*Apicystis bombi*	FN546182	100%	98.47%	*A. bombi*
			Apicystis bombi isolate 31	MZ379287	90%	99.74%	
			Apicystis bombi isolate 58	MZ379288	89%	99.34%	
Locustacarus buchneri	mtDNA CO1	OP741011	*Locustacarus buchneri*	AB052700	100%	98.16%	*L. buchneri*
			Locustacarus buchneri	AB052701	99%	97.60%	
			Locustacarus buchneri isolate Korea-1	MT309672	100%	97.42%	

Only one positive sample for each pathogen was sequenced and subject to Blast search [20]

1.20% for *C. bombi*, 1.20% for *A. bombi*, and 3.61% for *L. buchneri*. In commercial colonies, only *N. bombi* (9.52%) and *A. bombi* (19.04) were detected. In the case of screening *B. terrestris* samples from greenhouses, all pathogens (*N. bombi* (5.06%), *A. bombi* (1.26%) and *L. buchneri* (5.06%)) were detected at different rates, except for *C. bombi*. The infection rates in greenhouse samples were 5.06% for *N. bombi*, 0% for *C. bombi*, 1.26% for *A. bombi*, and 5.06% for *L. buchneri*. For the samples collected from nature, the infection rates were determined as 9.72% for *N. bombi*, 22.22% for *C. bombi*, 20.83% for *A. bombi*, and 5.55% for *L. buchneri*.

Co-infections were only detected in the samples collected from nature and the overall co-infection rate was 5.81%. The infection rate of the co-infections of *A. bombi* and *C. bombi* was 4.06% and the co-infection rate of *C. bombi* and *N. bombi* was 1.16%. Triple infection (*C. bombi*, *N. bombi*, and *L. buchneri*) was detected only in one sample (0.58%). A proportional diagram summarizing the infections and co-infections is given in Fig. 1.

The infection rates based on the provinces are given in Table 1. The Blast analysis results of each sequenced sample are given in Table 3. *C. bombi* ($Z = 3.78$, $p < 0.05$) and *A. bombi* ($Z = 4.47$, $p < 0.05$) infections were detected at higher rates in spring samples than in autumn samples. In terms of *N. bombi* ($Z = 0.16$, $p > 0.05$) and *L. buchneri* ($Z = 0.63$, $p > 0.05$) infections, there was no significant difference between the spring and autumn samples.

Discussion

Bumblebees are pollinator insects that are critically important in both natural and agricultural habitats and these bees are increasingly used in the pollination of various agricultural crops. Many recent studies showed that supporting the pollination of various plants with commercially produced bumblebees greatly benefits in pollen transfer and this eventually increases vegetable and fruit yields [21, 22]. However, the bumblebee populations have been decreased in many parts of the world [23] and this decrease is of great concern among scientists. In this sense, it is of great importance to clarify the factors that cause these declines. For this reason, in this study, it is aimed to investigate the presence, prevalence, and incidence of various pathogens that may cause declines in *B. terrestris* populations in Turkey.

In our study, the disease prevalence in *B. terrestris* populations was determined as 7.55% for *N. bombi*, 9.3% for *C. bombi*, 11.62% for *A. bombi*, and 4.65% for *L. buchneri*. When we look at the studies conducted in the world, it can be said that these disease rates in bumblebee populations vary according to regions. For example, in a study conducted

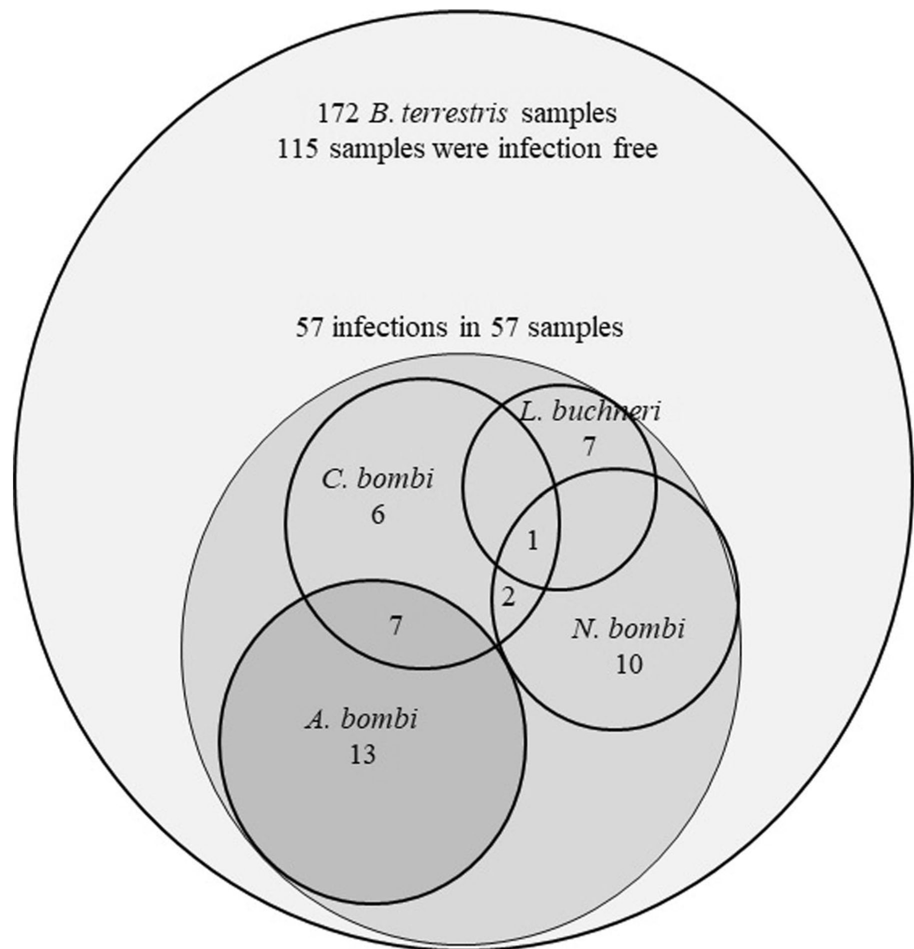

Fig. 1 A proportional diagram summarizing the infections and co-infections in 172 *B. terrestris* samples from Turkey. The large size of the circles represents the population size

in Slovenia, the presence of various pathogens in *Bombus* spp. workers was investigated by PCR and RT-PCR methods and *N. bombi* infection was found to be 16.3%, *N. ceranae* infection was 8.2%, *A. bombi* infection was 15%, and *C. bombi* infection was 17%. In queens, only *A. bombi* (26.3%) and *C. bombi* (33.3%) were detected [24]. In the study of Bosmans *et al*. [25], *N. bombi* and *C. bombi* were detected in *B. terrestris* queens by qPCR in Belgium at the rates of 8.3% and 43.8%, respectively. In the study of Felden *et al*. [26], the prevalence of *C. bombi* and *N. bombi* in *B. terrestris* populations was determined up to 100% and 30%, respectively, in different regions of New Zealand. In a study conducted in Spain, the prevalence of *N. bombi* in commercially produced *B. terrestris* populations was determined as 71% and *A. bombi* was detected in only 3 out of 919 samples. *L. buchneri* was not detected in any of the samples [27]. Yoneda *et al*. [28] investigated the presence of *L. buchneri* in four commercial *B. terrestris* colonies and found that the rate was 91.8% in worker bees, 73.9% in males, and 77.4% in newly emerged queens. Murray *et al*. [29] determined the pathogen prevalence in commercial *B. terrestris* colonies of Ireland as 61.8% for *N. bombi*, 35.3% for *Crithidia* spp., and 1.5% for *A. bombi*. *L. buchneri* and *Physocephala* sp. were not identified in any commercial colonies. They also found that 25% of the colonies were co-infected with *Crithidia* spp. and *N. bombi*.

Studies on the presence of pathogens in bumblebees in Turkey are quite limited. Eldeniz Cankaya and Kaftanoglu [16] detected *N. bombi* (12.96%), *C. bombi* (8.99%), *A. bombi* (6.89%), and *L. buchneri* (5.28%) infection in 578 *B. terrestris* queens collected from the Aegean and Mediterranean coastlines of Turkey. Aytekin *et al*. [17] investigated the presence of various pathogens in *B. terrestris* queens collected from Ankara province and its surroundings and they found that the infection rate was 20.9% for *Acarus farris* (Oudemans, 1905) (Acari: Acaridae) and 74.63% for *N. bombi*. When all these studies are examined, it is seen that the prevalence of the diseases in bumblebee populations vary according to factors such as the study region and the development stage of the bees. In this sense, to prevent the decreases in bumblebee populations, it is of great importance to investigate the presence of pathogens or parasites at regular intervals and in certain regions. This gives us the prominent information about the prevalence and incidence of the diseases to be investigated. During sampling, many factors such as climatic changes should be simultaneously

considered and the relationships between these factors and the incidence of diseases should be revealed.

Epizootics caused by insect pathogens are significantly influenced by environmental factors (biotic and abiotic), and in most cases, these factors such as sunlight, temperature, precipitation, and humidity are among the most influential factors in epizootic processes [30, 31]. These environmental factors (the weather parameters) are also known to influence the colony growth of bumblebees and the incidence of various pests and diseases [32–35]. For example, Sharma *et al.* [35] examined the seasonal incidence of various pests and diseases in *B. haemorrhoidalis* (Smith, 1852) (Hymenoptera: Apidae) colonies grown in the laboratory. As a result, it was determined that temperature and partial humidity directly affected the incidence of many pests and diseases during sampling and field establishment. In our study, *C. bombi* and *A. bombi* infections in spring samples were detected at the higher rate than in autumn samples. The reason for this is that environmental factors such as temperature and humidity could be more favorable for the development of diseases in the spring period and bees are more active especially in this period.

There are two main mechanisms (spillover and spillback) in terms of pathogen spread between managed and wild populations [40]. Different pollinator species such as honeybee and bumblebee collect pollen and nectar from flowers, and this allows pathogen transmission between them [40, 41]. Generally, the spread of pathogens occurs from honeybee and bumblebee farms to wild populations [7, 40, 41]. In this study, *N. bombi* and *A. bombi* were detected in three different populations (commercial, greenhouse, and nature colonies) of bumblebees. This is the most possible due to pathogens transmission between them, but this should be proved by molecular techniques such as DNA fingerprinting methods. In addition, in this study, some of the investigated pathogens (*N. bombi*, *C. bombi*, *A. bombi,* and *L. buchneri*) can also infect honeybees and this poses a potential risk to the health of honeybees and bumblebees both at colony and individual levels. Further detailed studies related to pathogen transmission between different populations including different species should also be conducted.

Consequently, the presence of four pathogens (*N. bombi*, *C. bombi*, *A. bombi,* and *L. buchneri*) in a total of 172 *B. terrestris* samples from Turkey was determined by PCR. The overall prevalence was determined as 7.55% for *N. bombi*, 9.3% for *C. bombi*, 11.62% for *A. bombi*, and 4.65% for *L. buchneri*. Ten samples were co-infected with at least one other pathogen. The infection rate of all examined pathogens appears to be relatively high to pose a risk in the investigated area. Therefore, necessary precautions should be taken to prevent pathogen transmission. A more detailed sampling strategy is needed to determine the transmission pathways of these disease agents between populations. In addition, pathogens in different populations need to be genotyped using the detailed molecular methods. Moreover, spillover and spillback of these pathogens from bumblebees to bumblebees or to honeybees should be investigated.

Author Contributions AS participated in almost all parts of the study such as the study conception, design, analysis, and writing the manuscript. RA participated in PCR and sequence analysis. SHÖ, FY, ÜK, HE, ÜK, MB, and ŞU performed collection of the bee samples and DNA extraction from the samples. Elif Sevim participated in gene sequencing.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

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