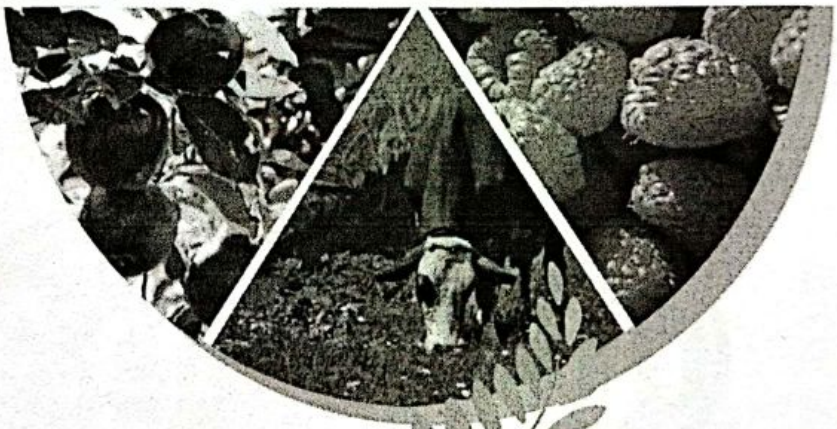
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MOLECULAR DETECTION OF PROTOZOAN PATHOGENS IN COMMERCIAL BUMBLEBEE (*B. terrestris* L.)

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Abstract

Bumblebees are important pollinators of greenhouse and field crops. They are therefore reared commercially on a large scale. *Bombus terrestris* is the most reared bumble bee species for commercial pollination since 1987 and has been used outside its natural distribution area. Commercially produced *B. terrestris* colonies often have higher levels of pathogens than wild bumble bee populations. These pathogens can spread to wild bees and may lead to decline in wild bee species. The most commonly reported bumblebee protozoan pathogens are the microsporodium *Nosema bombi, Crithidia bombi* and *Apicystis bombi.* These parasites have also detrimental effects on colonization, survival and reproduction of *B. terrestris* queens. Therefore, the correct identification of *B. terrestris* parasites is very important. Nowadays, in order to identify infected bumblebees, various molecular methods have been developed to provide highly sensitive, reliable and fast results. In this study, a total of 130 *B. terrestris* queens who died in the colony initiation period after diapauses were obtained from the commercial company. DNA was extracted from bumblebee gut tissues using a modified Chelex 100 method. ITSf2/r2, Nbombi-SSU-Jf1/Jr1 and SSU-rRNA-Ff1/Rr1b primer pairs for *N. bombi*, SEF/SER, CB-ITS1-F / CB-ITS1-R primer pairs for *C. bombi* and NeoF/NeoR, ApBF1/ ApUR2 primer pairs for *A.bombi* were tested by PCR amplification, but these parasites were not detected in the examined samples. In conclusion, more samples should be investigated to detect infected bumblebees with *N. bombi, C. bombi* and *A. bombi.*

Keywords: *Bumblebee, PCR, Nosema bombi, Crithidia bombi, Apicystis bombi*

Introduction

Bumblebees are among the most important natural pollinators in northern temperate and may provide superior pollination services than the honeybees for many plant species. There are about 250 species of bumble-bees (Williams, 1998). Currently five species of bumble-bees (*Bombus terrestris, Bombus lucorum, Bombus occidentalis, Bombus ignitus* and *Bombus impatiens)* are reared commercially on a large scale mainly for greenhouse pollination in many countries (Velthuis and van Doorn, 2006). The main commercially reared species is the Eurasian *Bombus terrestris* L. Commercial rearing of bumble bee colonies for pollination services first started in the 1980s. Today, it is estimated that the current worldwide sales of commercially produced bumble bee colonies has reached over one million colonies per year and has been used as pollinator of many crops mainly in tomatoes (Banda & Paxton, 1991; Velthuis and van Doorn, 2006).

Bumblebees are also important natural pollinators for a wide range of wild plants. On the other hand in recent years, the natural abundance and diversity of bumblebee species is in decline all over the world (Goulson, 2003). This decline may lead to severe effects on natural populations of flowering plants and agricultural production (Memmott *et. al.*, 2004). There are many factors regarding the causes of bumblebees decline such as climate change, habitat fragmentation with diminished floral resources and pathogens. The most important of these factors is considered as pathogens (van Engelsdorp *et. al.*, 2009; Cameron *et. al.*, 2011).

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Pathogens play a role in the decline of bumblebee species and can also affect the ecology and distribution of bumblebees (Schmid-Hempel, 2001; Cameron *et al.*, 2011). Pathogens have also caused economic losses in commercially managed bumble bees populations. Commercially reared bumblebee colonies can function as parasite reservoirs, which can lead to the spillover of pathogens to wild bee species. They are able to interact with wild pollinators, since commercially reared bees often escape from greenhouse to wild. A recent report by the National Academy Sciences stated that, a possible cause of bumble bee declines is pathogen spillover from commercially reared bumblebee colonies. Therefore, pathogens loads of commercially reared bee colonies could be important for wild bee species.

Many methods have been developed for the diagnosis of microsporidia in hosts, including immunofluorescent staining, electron microscopy, and antigenic or biochemical analysis (Weber *et. al.*, 1999). Molecular techniques, particularly involving the PCR and the based on the amplification of rRNA and rDNA gene fragments, seem to be superior because of their high specifity and sensitivity (Weiss and Vossbrinck, 1993) as well as their ability to detect all microsporidian developmental stages. Here, we used a rapid, specific and sensitive method for the detection of *N.bombi, C.bombi* and *A. bombi* in commercial reared *B. terrestris* by PCR amplification.

Bumble-bee parasites and diseases

Natural and commercially bumblebee colonies are exposed to many pathogenic internal and external parasites. The most commonly reported bumblebee protozoan pathogens are the microsporodium *Nosema bombi, Crithidia bombi* and *Apicystis.* Generally, these parasites have detrimental effects on colonization, survival and reproduction. Therefore, the accurately identification and control of bumble bee diseases and parasites are very important.

Nosema bombi Fantham and Porter 1914 is obligate, intracellular and spore forming microsporidian parasite that infects the Malpighian tubules, the ventriculus, the fat tissue and and even nerve tissues, including the brain of bumble-bees (Larsson, 2007). The effects are generally chronic, including reduction in individual reproductive rate (Otti and Schmid-Hempel, 2007, 2008), life span, and colony growth (Rutrecht and Brown, 2009).

Crithidia bombi is extracellular trypanosomatid parasites and it is a widespread parasite of bumble-bees. This parasite targets the gut and results in large numbers of parasite cells lining the wall of the midgut lumen and rectum from where cells are shed for further transmission (Schmid-Hempel, 1998). Replication of *C. bombi* in the host is rapid but the effects on individuals are usually complicated, such as decrease pollen loads carried in foraging trips, variation in foraging behavior and increase ovaries development in workers (Otterstatter and Thomson, 2006). Furthermore, on the colony level, *C. bombi* leads to decrease colony growth rate and colony fitness (Brown *et. al.*, 2003).

Apicystis bombi, first discovered in Italy in 1988, *is* a neogregarine parasite and has now been recorded in nearly 20 bombus species (Lipa and Triggiani, 1992; 1996) and commercially produced bumble-bee colonies (Murray *et. al.,* 2013). The fat body of infected bumble-bees is destroyed due to the proliferation of the pathogen, and its presence correlates with high mortality in infected spring queens, preventing them from establishing colonies (Rutrecht and Brown, 2008). Therefore, the potential spread of the neogregarine *A. bombi* should be carefully monitored.

Materials and Methods

Samples

We were collected 130 bumblebee queens from commercially produced Bombus *terrestris* colonies in different nests in order to investigate the occurrence of pathogenic diseases in august 2014. Samples of queen who died in the colony initiation period after diapauses were

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obtained from the commercial company. All samples were stored in 95% ethanol at -20 °C until DNA extraction.

DNA extraction

Genomic DNA of bumblebee was extracted from infected intestinal tissues using a modified Chelex 100 method (Walsh *et. al.*, 1991). All intestinal tissues of bumble-bee were put into large centrifuge tube. First, 800 µl 10% Chelex resin solution was added and completely crushed with sterile plastic types. Second, 10 µl Proteinase K (20 mg/ml) was added in each tube and vortexed. The sample was incubated at 56 °C for 4 h, followed by 95 °C for 15 min to denature enzymes. Samples were centrifuged (2 min, 12.000 g) and the supernatant was stored at -20 °C until needed as DNA template in PCRs.

Primers

rRNA primers of *Nosema bombi* were designed on conserved rRNA regions of microsporidian species. *Crithidia* and *Apicystis* primers were designed from the 18S rDNA sequences. An internal amplification control was designed; different 18S rDNA sequences Apidae species. (Table 1).

Table 1. List of oligonucleotide primer used in PCR

Primer's name	Sequence (5'→ 3')	T_a °C	Fragment size (bp)	References
Nbombi-SSU-Jf1-F Nbombi-SSU-Jr1-R	CCATGCATGTTTTTGAAGATTATTAT CATATATTTTTAAAATATGAAACAA TAA	50	323	Klee *et.al.*,2006
ITSf2-F ITSr2-R	GATATAAGTCGTAACATGGTTGCT CATCGTTATGGTATCCTATTGATC	48	118-122	Tay *et.al.*,2005
SSU-rRNA-Ff1 SSU-rRNA-Rr1b	CACCAGGTTGATTCTTCTGCCT TGTTCGTCCAGTCAGGGTCA	64	545	Tay *et.al.*,2005
Apidae-F Apidae-R	AGATGGGGGCATTCGTATTG ATCTGATCGCCTTCGAACCT	57	130	Meeus *et.al.*,2009
SEF SER	CTTTTGGTCGGTGGAGTGAT GGACGTAATCGGCACAGTTT	60	420	Meeus *et.al.*,2009
CB-ITS1-F CB-ITS1-R	GGAAACCACGGAATCACATAGACC GGAAGCCAAGTCATCCATCGC	55	276	Schmid Hempel and Tognazzo, 2010
NeoF NeoR	CCAGCATGGAATAACATGTAAGG GACAGCTTCCAATCTCTAGTCG	57	260	Meeus *et.al.*2009
ApBF1 ApUR2	CGTACTGCCCTGAATACTCCAG TTTCTCATTCTTCAGATGATTTGG	60	850	Meeus *et.al.*2009

Polymerase Chain Reaction (PCR)

PCR reactions were performed using a TC3000 (Techne) in 50 µl volume containing 5.0 µl of template DNA, 4 µl of 10x PCR buffer, 4 µl of $MgCl_2$, 2 µl of dNTPs, 0.2 µl of Taq polymerase and 0.25 µl of each forward and reverse primer, plus 34.3 µl of water. PCR parameters for amplification were as follows: initial DNA template denaturation at 95 °C for 4 min, 40 cycles of denaturation at 95 °C for 1 min, primer annealing at the primer specific temperature (Table 1) for 1 min and primer extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Then 10 µl of the PCR products were resolved on % 1.5 agarose gels (1x TBE) and visualized by ethidium bromide staining. For each PCR experiment,

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negative (water) and positive (previously identified positive sample) controls were run along with treatments.

Results and Discussion

We collected and analysed a total of 130 commercial *B. terrestris* queens in order to detect of microsporidia. Currently, many different primer pairs are available for the detection of *N. bombi, C. bombi* and *A. bombi* in host gut tissues. We tested seven different primer pairs (Table 1) but it did not happen in any amplification. The amplification occured only in the positive controls and Apidae primers.This result was mainly due to the small number of samples used in this study. Indeed, the number of samples used in previous studies was greater than 130. For example, Cordes *et al* (2012) analyzed 9909, Li *et al* (2012) 1009 and Blaker *et al* (2014) 1036 bumblebee samples. Therefore, it can be conclude that more samples should be used to analyses.

Tay *et al* (2005) characterized the entire rRNA gene of a *N. bombi* from 11 isolates in 8 different European *Bombus* species using 9 primer pairs including ITSf2- ITSr2 and SSU rRNA Ff1-Rr1b. Li *et al* (2012) screened a large sample of workers of 27 different Bombus spp. using SSU rRNA Ff1-Rr1b primer pairs in China and found that 13 different *Bombus spp.*, approximately half of the examined species, carried microsporidia. In North America, nearly 10,000 samples of 36 bumblebee species were collected and evaluated for the presence and prevalence of two *Bombus* pathogens, the *N. bombi* and *Crithidia* (Cordes *et. al.,* 2012). In this study, widely used primer sequences were tested.

PCR, qPCR and multiplex PCR based methods were developed for the specific and sensitive diagnosis of the microsprodian parasites in bumble bees. Klee *et al* (2006) conducted the first study of the specific and sensitive molecular diagnosis of *N. bombi* in bumble bees. To detect *N.bombi,* they used primer pair Nbombi-SSU-Jf1/Jr1, which amplifies partial SSU rRNA of the *N.bombi* genome. This primer pairs seems to be specific for *N.bombi.* Primer pair ITS-f2/r2 was extremely sensitive (as few as 10 spores per 100 µl homoganete) in the detection of *N. bombi* spores. This work is similar to the first comparative quantitative real-time PCR study of nine *Nosema spp.* primers within the framework of primer specifity and sensitivity (Erler *et. al.,* 2012). Another study was performed by multiplex PCR method. Meeus *et al* (2009) were reported the development of a multiplex PCR using two broad-range primer pairs for PCR- based protozoa detection including a universal Apidae control. In this study, SEF-SER, NeoF- NeoR and ApBF1- ApUR2 primer pairs were used for detection *C. bombi* and *A. bombi*, respectively.

Conclusion

In conclusion, PCR- based molecular methods and primers should be continuously developed and tested. Also, the screening of parasite in natural and commercial reared bumblebees should be done regularly.

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