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Molecular and Microscopic Detection of Microsporidia in Some Silkworm (*Bombyx mori* L.) Populations in Turkey

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Abstract

The silkworm, an easy to grow and quite precious insect, is affected by many diseases like other organisms. The microsporidia spores caused to Pebrine which is quite dangerous and causes serious economic damage. In this study, 450 silkworm eggs (*Bombyx mori* L.) collected from Bursa province in Turkey were fed into ten different groups until the 4th and 5th larval stages. Nine groups were fed with unwashed mulberry leaves whereas only one group was fed with washed ones. The samples at the 4th and 5th larval stages from each group were screened for *N. bombycis* and *Vairimorpha* sp. using both microscopic and molecular methods. There were no nosema spores detected in dyed samples under microscope (100Xobjective). The primers, specific for *N. bombycis* NBEF35F, NBEF957R and *Vairimorpha* sp. M1196F, M11822R classified into microsporidia, were used for detection of disease from isolated DNAs. There was no pathogens detected using molecular technique. In conclusion, considering that infection is due to horizontal transmission, the study results may demonstrate that food materials and silkworm from breeding region are safe for disease pathogens. This study is the first molecular application for detection of *N. bombycis* and *Vairimorpha* sp. in Turkey.

Keywords: Microsporidia, *Vairimorpha*, *Nosema bombycis*, PCR, Silkworm (*Bombyx mori* L.)

Türkiye'de Bazı İpek Böceği (*Bombyx mori* L.) Populasyonlarında Microsporadianın Moleküler ve Mikroskobik Tespiti

Özet

Yetiştirilmesi kolay ve oldukça kıymetli olan ipek böceği, diğer canlılarda da olduğu gibi birçok hastalıktan etkilenmektedir. Bunlardan birisi microsporadia sporlarının neden olduğu oldukça tehlikeli ve ciddi anlamda ekonomik zarara neden olan Pebrine hastalığıdır. Bu çalışmada, Bursa ilinden toplanan 450 adet ipek böceği (*Bombyx mori* L.) yumurtaları 4. ve 5. larval döneme kadar 10 farklı gruba ayrılarak beslenmiştir. İlk 9 grup yıkanmamış dut yaprakları ile beslenirken 1 grup yıkanmış dut yaprakları ile beslenmiştir. 4. ve 5. larval dönemdeki örnekler *N. bombycis* ve *Vairimorpha* sp. tespiti için mikroskobik ve moleküler metotlar kullanılarak taranmıştır. Boyanmış örnekler mikroskop (100Xbüyütme) altında incelendiğinde nosema sporuna rastlanmamıştır. İzole edilmiş DNA örneklerinde *Nosema bombycis'*e özgü NBEF35F, NBEF957R ve aynı zamanda microsporidia içinde yer alan *Vairimorpha* sp. M1196F, M11822R primerleri kullanılarak hastalık taraması yapılmıştır ve hastalık patojenine rastlanmamıştır. Sonuç olarak, horizontal bulaşma göz önüne alındığında, çalışma yetiştirmenin yapıldığı bölgenin ve yiyecek materyallerinin patojen bakımından güvenli olduğunu göstermiştir. Bu çalışma, *Nosema bombycis* ve *Vairimorpha* sp.' in belirlenmesine yönelik ilk moleküler uygulamadır.

Anahtar kelimeler: Microsporidia, *Vairimorpha*, *Nosema bombycis*, PZR, İpekböceği (*Bombyx mori* L.)

Introduction

The silkworm (*Bombyx mori* L.) is a kind of moth which can be fed on mulberry leaves that can live about 2 months. Although silkworm breeding is done in approximately 15 countries in the world, silk is used by many countries. Silk was produced firstly about 5000 years ago by the Chinese and after its method of production was kept as a secret for many years, it was first started to be produced in Anatolia and then in Europe. Silkworm breeding

goes back to 1500 years ago in Anatolia (Aydın et al., 2007; Şahan, 2011). Silkworms really contribute to the economy of the country both with the production of raw silk and the intensive use of the produced silk in textile industry. However, the production performance of silkworm is interrupted by diseases and pests like in other living things. One of the most important diseases is the pebrine caused by several kinds of microsporidia (Hatakeyama and Hayasaka, 2003; Aydın et al., 2007). This disease, which was first observed in the 19th century in Europe and caused serious losses, was observed in the hemolymph of silkworms and Balbiani (1882) described *Nosema* as microspora (Hatakayema and Hayasaka, 2002; 2003; Jyothi et al., 2005; Keeling and Fast, 2002; Patil et al., 2002; Singh et al., 2012). The disease spreads in two ways. The first is the Horizontal transmission which takes place orally during the eating of the mulberry leaves carrying *N. bombycis* spores by the silkworms and the other one is the Vertical transmission which takes place by the transfer of the *N. bombycis* spores in the ovary of the female moth to the future generations in a transovarial way. The epidemic and spreading of the disease cause decrease in the production of silk and significant economic losses as a result (Aydın et al., 2007; Hatakeyama and Hayasaka, 2002; 2003; Jyothi et al., 2005; Patil et al., 2002).

The disease can be observed in all of the development stages of the silkworm. The larvae die before they reach their last age periods. It was reported that the silkworms which reached the age of spinning cocoons could not spin cocoons when they were taken to the hanger and the larvae could not transform into the state of complete chrysalis (Aydın et al., 2007; Hatakeyama and Hayasaka, 2002; 2003; Jyothi et al., 2005; Singh et al., 2012). When the spores are observed in the population, the disease is already spread; it causes losses in the silkworms who reach to this stage. In Turkey, it was first seen in 1860s in Bursa (Aydın et al., 2007). Disease causes serious silkworms losses and decrease in the amount of silk produced when regarded from the point of view of silk production industry. Because of this reason, the losses will decrease with the diagnosis of *N. bombycis* and *Vairimorpha* sp. at an early stage or the prevention of the factors of the disease before they spread. It was done on cases which were informed with the microscopic diagnosis of *N. bombycis* on the suspicious samples in Turkey (Aydın et al., 2007). However, the nosema spores can be overlooked under microscope, they are very small (3.4 -3.8 µ height and 2.0- 2.3 µ width) and it was also difficult to observe under microscope (Iwano and Ishihara, 1981; Kawarabata and Ishihara, 1984).

It propounds that molecular diagnosis is very important for the determination of *N. bombycis* and *Vairimorpha* sp. in an early period. The spores of *N. bombycis* do not reveal themselves for a long time if a suitable environment does not form and moreover, even if the spore is in the environment, the disease does not form (Undeen and Solter, 1996). At this point, it was tested that if the leaves collected from different areas contained nosema spores, do the spores during their feeding periods with the leaves infect to silkworms in breeding regions? In accordance with the question, the microsporidia spores were scanned with microscopic and molecular methods in silkworms separated in groups according to feeding types.

Materials and Methods

450 silkworm (*Bombyx mori* L.) egg samples were collected from Bursa region (Longitude 29°01.8'E and Latitude 40°10.8'N) in Turkey in May 2013. The collected samples were brought to the laboratory in Kırşehir and separated into 10 different groups according to collecting sites. The larvae were fed until 4th and 5th larval stages. The mulberry leaves in order to feed the larvae were randomly collected from different locations in Kırşehir. The first 9 groups were fed with unwashed mulberry leaves, 1 group was fed with washed mulberry leaves. When the samples reached to 4th and 5th larval stages, stomachs and intestines of silkworm larvae were smashed in physiological saline solutions in order to get homogenates. The spore dyeing was done from homogenates from each group for microscopic diagnosis. Samples were dyed with 1% of the safranin and examined under X100 light microscope with the immersion oil for the microscopic diagnosis of spores (Aydın et al., 2007). DNA isolations were done from the prepared homogenates using DNA isolation kit (Fermentas K512). The isolated DNA samples were run on 1% agarose gel. Multiprimer sets NBEF35F (5-TGG CGC TGT TGA TAA GAG ATT-3), NBEF957R (5-AAT TTA GCA ACA CAA GCC TTA T-3) for *N. bombycis,* M1196F (5-CTC GAA TTA GAA AAT TCT CTC AA-3) and M11822R (5-TAC TTT ATT TAA TGT ACA TTT GAA AA-3) for *Vairimorpha* sp. microsporadia were used in the PCR (Hatakeyama and Hayasaka, 2003). 25 µl PCR mixture that contained 0.5 µM each of primers, 0.25 mM of dNTPs, 1× Taq PCR buffer, 2.5 mM MgCl2, 0.5 units Taq DNA polymerase and 125 ng DNA was used. The PCR procedure started for an initial 2 minutes at 94°C , followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 40 s at 72°C, followed by a final extension of 5 min at 72°C. PCR products were

electrophoresed through 1.2% agarose gel with 100 bp DNA ladder, stained with REDGEL, and visualized using UV illumination (Ravikumar et al., 2011).

Results and Discussion

It was reported that the microsporidia contaminated to the silkworm, mulberry leaves or breeding beds in different ways, moreover, other insects carrying the disease spores were effective in the spreading of the disease, in the other studies done (Singh et al., 2012). In this study, the nosema spore which caused the disease could not be found in the silkworms generally separated to two feeding groups with washed and unwashed mulberry leaves and totally 10 groups including all insects for the determination of the microsporidia spores.

As for Turkey, two different cases were reported about pebrine disease caused by microsporidia from suspicious populations. According to one of these, it was reported that all of the eggs and the breeding lines produced in 2002 were annihilated by burning because they had Pebrine, according to the data from the Ministry of Food, Agriculture and Stockbreeding (Aydın et al. 2007). The other study was reported that the suspicious samples taken from the provinces Hatay, Bilecik and Bursa by the Ministry of Food, Agriculture and Stockbreeding in the spring period of 2004 with the Pebrine were microscopically examined by Aydın et al. (2007) for *N. bombycis* and Pebrine disease was found. It was reported that the microsporidia is difficult to observe under microscope and it could be overlooked because of its dimensions' being too small, in the microscopic diagnosis (Iwano et al., 1981 and Kawarabata and Ishihara, 1984). For that reason, there were many studies in order determine microsporodia from different ages silkworm. According to the studies done before, it was reported that the diseases could be diagnosed using immuno peroxidase staining method (Han and Watanabe, 1987), fluorescent anticore technique (Sato et al., 1982; 1981) and latex fluorescent anticore technique from mother moths (Hayasaka and Ayuzawa, 1987).

It is reported that it can also be diagnosed from the eggs of the silkworms using multiplex PCR in addition to this method, with the examination of the pieces directly taken from the larva or the pieces of feces, with the centrifugal method of the spores, in the years afterwards (Hatakeyama and Hayasaka, 2003; Patil et al., 2001; Saharan et al., 1992). It was reported that either *N. bombycis* among the microsporidia or *Vairimorpha* sp. spreaded more frequently in a transovarial than horizontal transmission and they could easily be determined at the egg phase of the silkworm in the determination of the disease using PCR (Han and Watanabe, 1987; Hatakeyama and Hayasaka, 2002).

In this study, any pathogens (*N. bombycis* and *Vairimorpha* sp.) caused to pebrine from samples were found with the multiplex PCR technique. In conclusion, our question was tested during in this study and there were no pathogens detected using molecular and microscopic methods. Considering that infection is due to horizontal transmission, the study results may demonstrate that food materials and silkworms from breeding region are safe for disease pathogens.

At the same time the present study showed its practicability in the diagnosis of the disease at the molecular level and also this is the first molecular application for detection of *N. bombycis* and *Vairimorpha* sp. in our country; however there were no pathogens found that caused disease from studied samples. It is thought that it will play an active role in the diagnosis and even the treatment of the disease in the early period together with the molecular techniques used in the increase of the knowledge about the silkworms which are infected with microsporidia and their spores, in the near future.

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