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Integration of Molecular and Genetic Methods in the Investigation of the Biodiversity of the Honey Bee (*Apis Mellifera L.*, Hymenoptera: Apidae) and Diagnosis of Diseases of Economic Importance Affecting the Bees and their Brood in Bulgaria

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Abstract

The Western honey bee (*Apis mellifera L., Hymenoptera: Apidae*) is species of crucial economic, agricultural and environmental importance. According to Albert Einstein "If the bee disappeared off the surface of the globe then man would only have four years of life left. No more bees, no more pollination, no more plants, no more animals, no more man". First domesticated around 5000 BC, nowadays the species has a worldwide distribution (excluding the Antarctic). Based on morphometrical, behavioral and biogeographical data, 29 subspecies of *A. mellifera* are recognized, also known as "geographical races", since their distribution corresponds to specific geographical areas. In the last few years a global decrease of honey bee hives is observed (from 21 mln to 15.5 mln) which is detrimental both to the production of honey bee products and to the pollination and production of many crops and wild plants. Reasons for this decline are of different origin and are not entirely understood but are linked to the synergistic effects of infestation with different parasites (varroosis and nosemosis), viral and bacterial infections (foulbrood) and widely used pesticides in the farming industry (including in genetically modified crops) as well as with the loss of genetic diversity.

Keywords: *A. mellifera*; Biomonitoring; Genotyping; Foulbrood; Varroosis; Nosemosis; Viral diseases

Current State of the Scientific Research on the Project Subject in Bulgaria and Abroad and Actuality of the Research Topic

The honey bee *A. mellifera* is well adapted to diverse ecological habitats. The species is cosmopolitan, found in its natural habitat in Africa, Europe, West and Central Asia and successfully introduced in early 1600s into the New World [1]. Bee populations and their saving are a priority of utmost economical, agronomical and ecological importance. Based on morphological, behavioral and molecular data, 29 subspecies of the honey bee are described [2]. They are grouped into 4 lineages with specific geographical distribution: African (A), West and North European (M), Southeastern European (C) and Oriental (O) [3,4].

Bee populations in Bulgaria belong to evolution line lineage C, in which 5 subspecies are included - *A. m. carnica, A. m. macedonica, A. m. ligustica, A. m. cecropia* and *A. m. sicula* [5]. The first two subspecies are typical for the Bulgarian fauna. With the intention of improving the productive qualities of Bulgarian bee families, in Bulgaria were introduced the subspecies *A. m. ligustica and A. m. caucasica* [6].

The genetic introgression between the native and the introduced subspecies is not investigated to the current moment. In Bulgaria, it is also present the indigenous subspecies *A. m. rodopica*, with undetermined taxonomical status. Some authors place it as a separate subspecies [7], while others include it into *A. m. carnica* [8] or *A. m. macedonica* [9].

The biodiversity of the honey bee in Bulgaria was determined and investigated on the basis of morpho-etiological, biochemical (aloenzyme markers) and genetic (microsatellite analysis, RAPD, mtDNA-RFLP analysis) methods [7,9].

The genetic and haplotype structure of the honey bee populations in Bulgaria up to date are not fully known. There is no data about the phylogenetic and evolutionary relationships within and among the different subspecies of *A. mellifera*.

A lot of factors lead to the dramatic diminution of bee populations, reduction of bee production and thus disruption of the process of pollination of farming crops and wild plants (including certain endemic ones).

Among such factors are known diseases with economic significance affecting the bees and their brood, such as viruses, bacterial (European and American foulbrood) fungal (chalkbrood and stonebrood) and parasitic (varroosis and nosemosis) [10]. The wide use of insecticides and herbicides in crop protection has on one hand a negative impact on ecological balance and on the other hand inflict heavy losses on the bee colonies. Adoption of genetically modified organisms and transgenic farming also impacts negatively the development and the productivity of bee families. Not least the anthropogenic factor (inbreeding, high voltage power lines, TV and cellular radio towers, errors in breeding techniques) also has a negative influence. All these factors lead to the so called Colony Collapse Disorder (CCD). Research indicates that only for year 2008 45% of bee families in the USA and 29% of bee families

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in Canada for the year 2007 have disappeared. In recent years this phenomenon has appeared in Europe too – in Spain, Greece, France, Switzerland, Germany and other countries [11].

The varroosis is the most important invasive honey bee disease caused by mites from *Varroa* genus. It inflicts seriously economy loses worldwide [12]. It is considered that varroosis became a problem after the invading of *A. mellifera* colonies by the *V. destructor* mite. The *V. jacobsoni* was the first described agent that causes the mentioned above disease in Eastern honey bee *A. cerana* and switches its host with Western honey *bee A. mellifera*. The *V. destructor* was previously considered to be *V. jacobsoni* species, but molecular based on mtDNA proved the presence of separate species [13]. For understanding the phylogeny and taxonomy status of Varroa species multiple geno- and haplotyping investigations were performed using different molecular-biological approaches: RAPD [14], PCR-RFLP of cox1 mitochondrial gene [12,15], as well as direct sequencing analysis [16]. Except cox1 gene other mitochondrial markers were also utilized for haplotypes determination of *Varroa* representatives - cox3, atp6 and cytb [16,17]. Genetic variability detection was performed also based on microsatellite marker profiles [15,18]. *Varroa* species are the main vectors for horizontal transfer of viruses in bee colonies [19-21] and a possible vectors for horizontal transmission of some bacteria (Wolbachia) [22]. Because of that most of the researches are focused on molecular identification of viral infection and their association with mites as vectors.

Up to date there are no molecular methods included in beekeeping industry for genotyping of *Varroa* genus, neither for their viral diseases relations.

Viral diseases affecting the honey bee also inflict heavy losses on the beekeeping industry. To the present moment at least 19 viruses infecting the honey bee are known, most importantly such as Acute Bee Paralysis Virus (ABPV), Kashmirbee Virus (KV), Cloudy Wing Virus (CWV), Black Queen Cell Virus (BQCV), Chronic Paralysis Virus (CBPV not fully taxonomically classified) [13], and Israeli Acute Paralysis Virus (IAPV). They are all picornalike (RNA) viruses belonging to families Iflaviridae and Dicistroviridae, and it is believed that their most important vectors are the ecto- (*Varroa*) and endoparasites (*Nosema*) of bees. Other viruses infecting the honey bee belong to the families Iridoviridae [23] and Secoviridae [24]. Some of these viruses (especially in coinfections with parasites are linked to the CCD).

There is neither available data for Bulgaria about the extent of the spread of viral pathogens in bee colonies nor data about the presence of co infections in bee colonies.

Global data demonstrates that mainly 3 viruses are connected with significant bee colony losses – CWV, ABPV and IAPV. The first one was initially connected to CCD [25], but at a latter stage it was concludes that the reasons for CCD are more complex. In Europe ABPV and IAPV are indicated as one of the reasons for the reduction of bee families in post winter season [23]. Both viruses are transmitted by the *varroos*is causative agent – the *varroa* mites invading the pupae or adult bees. ABPV is highly virulent, because it's located in the hemolymph. IAPV is less virulent, causes deforming of the wings and is observed in colonies with more severe *varroa* infestation. Despite its lesser virulence, IAPV infects different tissues of the honey bee, which impacts negatively the physiological functions of *A. mellifera* [26].

The European foulbrood is a bacterial disease affecting the brood, caused by the gram-positive bacterium *Melissococcus plutonius*. In infects mainly larvae at 3 or 4 day age. Often the infection is in fact a co infection since other microorganisms are reported also - *Enterococcus faecalis, Achromobacter euridice, Paenibacillus alvei* and *Brevibacillus laterosporus*. The disease has a worldwide distribution with significant economic importance in the Americas, Europe, Australia, India and South Africa [27].

Diagnosis of this disease is carried mainly through microscopic examination of the brood, cultivation of the pathogen in vitro, ELISA and LFI methods.

Molecular methods are also developed for the identification of *M. plutonius*, and the most often used are 16S DNA markers.

The American foulbrood is highly contagious disease affecting the brood, which inflicts severe economic losses on the beekeeping industry worldwide. The pathogen is a gram positive, spore forming bacterium *Paenibacillus* larvae [28]. Traditionally the diagnosis of this disease is based of its specific clinical manifestation. The American foulbrood is highly contagious disease affecting the brood, which inflicts severe economic losses on the beekeeping industry worldwide. The pathogen is a gram positive, spore forming bacterium *Paenibacillus* larvae [28]. Traditionally the diagnosis of this disease is based of its specific clinical manifestation and cultivation of the pathogen from infected bee colonies. In the recent years as alternative to the classic approach, molecular methods start to come into use and characterization and quantitative assessment of P. larvae. Conventional PCR (16S rDNA) for detection of the pathogen is the most widely used method in this kind. Real-time PCR is also a widely used technique for quantitative analysis of the causative agent within the honey or in the brood. For the epidemiological studies it is obligatory not only to assess the vegetative form of the pathogen, but also the spores in different biological material – honey, brood, bees and beeswax. Because of this molecular methods are developed for the isolation of genetic material from the endospores of *P. larvae* [29].

To the present moment mainly two species of microsporidia are known to infect the honey bee - Nosema apis, which parasitizes on the European (western) honeybee (*A. mellifera*), while *Nosema ceranae* is a pathogen found infecting the Asiatic (eastern) honeybee (*A. cerana*). Recently *Nosema ceranae* was introduced to different regions of Europe, the Americas, and North Africa and assumes the role of a dominant species [28].

Owning to its high virulence *N. ceranae* is causing severe economic losses on the beekeeping. Different geographically distinct strains of *N. ceranae* are in existence and it's assumed that they have different degrees of pathogenicity on the bee colonies [30]. On the Balkan Peninsula *N. apis* was considered the only Nosema species parasitizing on the honey bee until year 2007 [24].

There is no available data about Bulgaria concerning the occurrence of the two agents of nosematosis. It is not known if the highly virulent *N. ceranae* had replaced *N. apis* or if a mixed invasion has developed in some the bee colonies. Owning to the fact that the clinical manifestation of the disease is not always specific, several approaches are developed for its diagnosis. Both classical (microscopic examination of the spores) and molecular methods are applied. For the identification and differentiation of the two nosema species, and also for their quantitative determination are used PCR, PCR-RFLP, Real-time PCR and multiplex PCR method [31,32].

The detection and the determination of the genetic diversity, virulence and the pathological effects of parasitic, viral, fungal and bacterial honey bee diseases by means of molecular methods are not widely used and integrated in Bulgaria, with the exception of the

species identification of the nosematosis causative agents (*N. apis* and *N. ceranae*).

The adaptation of similar approach in Bulgaria will contribute to a wide spectrum of monitoring, diagnosis and management of these diseases [33].

The current project is aimed at widening the deepening of the existing knowledge about the biodiversity of the bee populations and monitoring of the honey bee diseases of economic importance. Project results will contribute to the integration within the beekeeping practice of molecular and genetic methods for the diagnosis and control of diseases causing losses to the bee families. Breeding programs will be implemented aimed at specific bee colonies, resistant to certain pathogens.

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