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Variability of the honey bee mite *Varroa destructor* in Serbia, based on mtDNA analysis

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Abstract Only two mitochondrial haplotypes (Korea and Japan) of *Varroa destructor*, the ectoparasitic honey bee mite, are known to be capable of infesting and successfully reproducing in *Apis mellifera* colonies worldwide. *Varroa destructor* (then called *Varroa jacobsoni*) was observed in Serbia for the first time in 1976. In order to obtain insight into the genetic variability of the mites parasitizing *A. mellifera* we analyzed 45 adult female mites sampled from nine localities dispersed throughout Serbia. Four fragments within *cox1*, *atp6*, *cox3* and *cytb* mtDNA genes were sequenced. The Korea haplotype of *V. destructor* was found to be present at all localities, but also two new haplotypes (Serbia 1 and Peshter 1) were revealed, based on *cox1* and *cytb* sequence variability. The simultaneous occurrence of Korea and Serbia 1 haplotypes was observed at five localities, whereas Peshter 1 haplotype was identifed at only one place.

Keywords *Varroa destructor* · Mitochondrial DNA · Haplotype · *Apis mellifera* · Serbia

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Introduction

Varroa destructor Anderson and Trueman, an obligate ectoparasitic mite of honey bees, is a major threat for apiculture worldwide (Rosenkranz et al. 2010). This parasite species has shifted from its original host, Eastern honey bee (*Apis cerana* Fabricius) to a new host, the Western honey bee (*Apis mellifera* L.) and has become cosmopolitan. After a detailed study of the morphology and genotype of many *Varroa* mite samples collected worldwide, Anderson and Trueman (2000) described *V. destructor* as a new species parasitizing both *A. cerana* and *A. mellifera*. Before 2000 most of the scientific literature referring to *V. jacobsoni* in fact dealt with *V. destructor*; *V. jacobsoni* only parasitizes *A. cerana*. Nowadays, *V. destructor* is considered to be the main cause of winter losses of *A. mellifera* colonies (Genersch et al. 2010; Guzmán-Novoa et al. 2010).

The first variability findings concerning the honey bee mite, then known as *V. jacobsoni*, were obtained using morphometric and biochemical analyses (Grobov et al. 1980; Delfinado-Baker and Houck 1989; Issa 1989; Nation et al. 1992). Variability at the DNA level was studied by RAPD, RFLP and sequencing methods (Kraus and Hunt 1995; de Guzman et al. 1997, 1998; Anderson and Fuchs 1998). Anderson and Trueman (2000) explored the variability of a 458 bp sequence of mitochondrial cytochrome oxidase 1 (*cox1*) from *V. destructor* and *V. jacobsoni* mites sampled from *A. cerana* and *A. mellifera* colonies worldwide and found 18 mitochondrial haplotypes. Nine of them belonged to *V. jacobsoni*, six of them belonged to the newly described *V. destructor* species, whereas the other three haplotypes remained unclassified. However, only the Korea (K) and Japan (J) types (named after the countries where they were first detected parasitizing *A. cerana*) successfully colonized *A. mellifera*. Haplotype K is spread throughout the world (Anderson 2000; Anderson and Trueman 2000; Solignac et al. 2005), whereas J has been found in Japan, Thailand and the Americas (de Guzman et al. 1999; Anderson and Trueman 2000). These haplotypes are two partly isolated clones with almost no polymorphism due to the genetic “bottleneck” that mites went through at the time of host change (Solignac et al. 2005). Revealing the complete sequence of the *V. destructor* mitochondrial genome was of great importance for further studies of the mite’s genetic variability (Evans and Lopez 2002; Navajas et al. 2002). A new haplotype of *V. destructor* was found on *A. cerana* in China (Zhou et al. 2004), whereas four new haplotypes were discovered on *A. mellifera* throughout Asia, using new, more sensitive mtDNA markers (Navajas et al. 2010).

After the first detection of honey bee mites in Serbia in 1976 (Lolin 1977) only the K haplotype of *V. destructor* was identified among samples from former Yugoslavia (Anderson and Trueman 2000). Recently, preliminary mtDNA analyses of *V. destructor* from Serbia indicated variability within the *cox1* gene (Gajic et al. 2011). The aim of this study was to explore mtDNA variability of *V. destructor* in *A. mellifera* colonies from Serbia in more detail.

Materials and methods

Mite collection

A total of 45 adult *V. destructor* females were sampled from *A. mellifera* colonies in nine localities from different geographical regions of Serbia (Fig. 1). In each locality, samples were collected from one apiary. Mites were collected from the hive bottom boards 24 h after acaricide treatment and were stored in 70 % ethanol until needed for DNA extraction.

Archival samples (from Vrbica, Bor and Zajecar) were collected as already described, and they had been stored permanently at ambient temperature with no preservatives (Table 1).

DNA extraction

Total DNA was extracted from single females using the Kapa Express Extract kit (Kapa Biosystems, USA, Cat. No. KK7103) following the manufacturer's instructions. Extracted DNA was then diluted (1:5) in 1× TE buffer (Serva Electrophoresis GmbH, Germany, Cat. No. 39799.01) and immediately used for PCR or stored at −20 °C. The same extraction method was applied for all samples.

PCR amplifications

Fragments of four mitochondrial genes were amplified: *cox1*, cytochrome oxidase 3 (*cox3*), ATP synthase 6 (*atp6*) and cytochrome b (*cytb*) using previously designed primers (Solignac et al. 2005; Navajas et al. 2010) (Table 2). Two fragments within the *cox1* gene that differed in length (the shorter of 376 bp and the longer of 929 bp) and partially overlapped were amplified using two pairs of primers. The fragment containing parts of both, *atp6* and *cox3* genes, was amplified using one pair of primers (Table 2).

Fig. 1 Geographical distribution of *Varroa destructor* sampling localities in Serbia (*1* Palic, *2* Belgrade, *3* Vrbica, *4* Bor, *5* Zajecar, *6* Boljevac, *7* Zlatibor, *8* Suvi Do, *9* Saprance)

Table 1 Collecting sites for *Varroa destructor* mites in Serbia

No.	Locality	Years	Coordinates	Altitude (m)
1	Palic	2011	46°06′32″N, 19°46′02″E	103
2	Belgrade	2011	44°49′07″N, 20°28′05″E	99
3	Vrbica	1992	44°17′16″N, 20°35′02″E	250
4	Bor	2000	44°04′42″N, 22°05′43″E	393
5	Zajecar	2001	43°54′15″N, 22°17′05″E	128
6	Boljevac	2011	43°49′49″N, 21°57′11″E	283
7	Zlatibor	2011	43°43′24″N, 19°42′15″E	977
8	Suvi Do	2011	43°02′43″N, 20°07′15″E	1179
9	Saprance	2011	42°23′21″N, 22°00′17″E	560

Table 2 Primer sets used for PCR amplification of gene sequences

Gene	Size (bp)	Primer name	Primer sequence (5′→3′)	Ta (°C)	References
cox1[a]	376	COI376bpF COI376bpR	TACAAAGAGGGAAGAAGCAGCC GCCCCTATTCTTAATACATAGTGAAAATG	52	Solignac et al. (2005)
cox1	929	10KbCOIF1 6,5KbCOIR	CTTGTAATCATAAGGATATTGGAAC AATACCAGTGGGAACCGC	51	Solignac et al. (2005), Navajas et al. (2010)
atp6-cox3	818	16KbATP6F 16KbCOIIIR	GACATATATCAGTAACAATGAG GACTCCAAGTAATAGTAAAACC	51	Solignac et al. (2005), Navajas et al. (2010)
cytb	985	10KbCytbF-1 10KbCytbPRIM	GCAGCTTTAGTGGATTTACCTAC CTACAGGACACGATCCCAAG	52	Solignac et al. (2005), Navajas et al. (2010)

[a] Sequence partially included in the 929 bp *cox1* sequence used for comparison with the published haplotypes of Anderson and Trueman (2000)

PCR was carried out in 25 μl volume containing 12.5 μl 2× Kapa2G Robust HotStart ReadyMix (Kapa Biosystems, USA, Cat. No. KK5702), 0.5 μM of each oligonucleotide primer and 5.0 μl diluted DNA template. The amplification cycle consisted of an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 15 s denaturation at 95 °C, 30 s annealing (the annealing temperature for each fragment is given in Table 2) and 1 min extension at 72 °C. The final extension lasted for 2 min at 72 °C. PCR was performed in a MultiGene™ Gradient Thermal Cycler (Labnet International, USA). Amplification efficiency was controlled by electrophoresis of PCR products in 2 % agarose gel stained with ethidium bromide and visualized under UV light.

Sequence analyses

Positive PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA, Cat. No. 28104) and directly sequenced in two directions using the BigDye®

Terminator method in an ABI 3730XL automatic DNA sequencer (Macrogen Europe, The Netherlands).

All obtained sequences were compared with the complete mitochondrial sequence of *V. destructor* (GenBank Accession Number AJ493124.2, Navajas et al. 2002), as well as with appropriate individual sequences of the K haplotype obtained in previous studies (Anderson and Trueman 2000; Navajas et al. 2010). Sequences were analyzed using BioEdit version 7.0.9.0. (Hall 1999) and Clustal W software (Thompson et al. 1994).

Results

The obtained sequences revealed that 26 samples from 9 localities were identical to the K haplotype of *V. destructor* described by Anderson and Trueman (2000) (GenBank Accession Number AF106899). In 17 samples from five localities, including one sampled in 1992, variability was observed in the 929 bp *cox1* sequence at position 1932 of mtDNA. Mites which contained this unique *cox1* sequence were assigned to the new haplotype named Serbia 1 (S1) (Tables 3, 4). The same samples showed identical nucleotide position variability within the 376 bp *cox1* sequence. Only the 376 bp *cox1* fragment was successfully amplified from archival samples (localities Vrbica, Bor and Zajecar). Although shorter than the reference sequence (GenBank Accession Number AF106899, 458 bp long), our 376 bp *cox1* sequence was entirely situated within the reference one and contained enough informative sites for adequate comparison. Sample analysis showed

Table 3 Number of *Varroa destructor* individuals of a particular haplotype detected in the observed localities

Locality	No. of sampled bee colonies/mites	No. of individuals of defined *V. destructor* haplotype		
		Korea[a]	Serbia 1[b]	Peshter 1[c]
Palic	3/6	6		
Belgrade	6/10[d,e]	4	6	
Vrbica	2/3[e]	2	1	
Bor	1/1	1		
Zajecar	1/1	1		
Boljevac	3/6[d,e]	3	3	
Zlatibor	3/6[e]	2	4	
Suvi Do	3/6[f]	4		2
Saprance	3/6[d,e]	3	3	

[a] *cox1* sequences (376 bp) identical to the previously described Korea haplotype (GenBank accession number AF106899)

[b] New haplotype (variability within *cox1* sequence)

[c] New haplotype (variability within *cytb* sequence)

[d] Intrapopulation variation detected

[e] Interpopulation variation detected

[f] Sequences containing the variable nucleotide position for determination of the Peshter1 haplotype were sequenced for only two mite samples. *Cox1* sequences of the remaining *V. destructor* mites were identical to the Korea haplotype

Table 4 Nucleotide polymorphisms within *cox1* and *cytb* mtDNA sequences of *Varroa destructor*

Haplotype		Variable site				
		cox1				*cytb*
From previous study[a]	From current study	1731	1791	1932	2244	10133
K1-1		A	T	A	A	G
K1-2		.	.	.	.	.
K1-3		G	A	.	.	.
K1-4		.	.	.	G	.
	S1	.	.	G	–[b]	.
	P1	.	.	.	–[b]	A

Dots indicate an identical nucleotide as in the first sequence. Numbers correspond to nucleotide positions in the complete mitochondrial sequence of *V. destructor* (GenBank Accession Number AJ493124.2; Navajas et al. 2002)

[a] Navajas et al. (2010)

[b] This nucleotide position is not included in our *cox1* sequence

Table 5 GenBank accession numbers of *Varroa destructor* mtDNA sequences compared

Haplotype	GenBank accession numbers			
	cox1	*atp6*	*cox3*	*cytb*
K1-1[a]	GQ379056	GQ379113	GQ379075	GQ379094
K1-2[a]	GQ379057	GQ379114	GQ379076	GQ379095
K1-3[a]	GQ379059	GQ379116	GQ379078	GQ379097
K1-4[a]	GQ379060	GQ379117	GQ379079	GQ379098
S1[b]	JX970938	JX970940	JX970942	JX970944
P1[b]	JX970939	JX970941	JX970943	JX970945

[a] Haplotypes assigned to the K haplogroup according to Navajas et al. (2010)

[b] New haplotypes obtained in the current study

simultaneous existence of the K and S1 haplotypes at five localities, both within the same hives (intrapopulation variation) and within various hives at the same collecting site (interpopulation variation) (Table 3). In two samples from Suvi Do locality a variable nucleotide was observed within the *cytb* sequence at position 10,133 of mtDNA. These mites were assigned to another new haplotype of *V. destructor* and named Peshter 1 (P1) (Tables 3, 4). Both described *cox1* and *cytb* single nucleotide variations were "silent" mutations that did not affect protein sequences. There were no samples with simultaneous nucleotide variability in *cox1* and *cytb*. All *atp6-cox3* sequences were identical to previously published sequences of the K haplotype. GenBank accession numbers for the mtDNA sequences of the new haplotypes described in the current study are given in Table 5 together with others for comparison.

Discussion

In this study, fragments of *V. destructor cox1*, *cox3*, *atp6* and *cytb* mtDNA genes were sequenced to analyze genetic variability of this parasite in *A. mellifera* colonies in Serbia. The presence of the *V. destructor* K haplotype described by Anderson and Trueman (2000) was confirmed in all investigated localities. In addition, two new haplotypes were discovered, Serbia 1 (S1) and Peshter 1 (P1). This is the first report of new *V. destructor* haplotypes in Europe. Compared to the K haplotype, the S1 haplotype has a single point mutation in the *cox1* sequence at position 1932. The same mutation was detected in both the short and long sequence of *cox1* analyzed here, leaving no doubt about this finding. Since the same haplotype was identified in a sample collected in 1992, it is likely that it has been present in Serbia for at least two decades. However, due to the lack of data in the literature, it is not possible to determine whether the S1 haplotype was introduced to Serbia or it resulted from a new mutation.

Haplotypes K and S1 were found together in five localities. In two of them, these haplotypes occurred only in different hives. However, in three localities, both haplotypes were identified not only in different hives but also in the same hive. This contrasts with previous findings of only one haplotype of *V. destructor* on *A. mellifera* per locality (Navajas et al. 2010; Maggi et al. 2012). However, de Guzman et al. (1999) detected both K and J haplotypes of *V. destructor* at one locality, both within the same and in different hives. The intra-hive variability detected in our study might be the consequence of beekeeping practices in Serbia. High density of hives during the pasture season contributes to easier spread of a new haplotype by robbing activities or by drifting of foragers (Frey et al. 2011). Also, these results indicate that collecting a single mite per colony might introduce a bias in estimates of genetic variation.

The detection of only one mite of the J haplotype together with many *V. destructor* mites of the K haplotype in the same apiary was described by Muñoz et al. (2008) and interpreted as the result of uncontrolled importation of honey bee queens from the American continent. However, the simultaneous existence of K and S1 haplotypes of *V. destructor* in different localities in Serbia, as well as the finding of the S1 haplotype in the sample from 1992 point to a well established infestation with this newly described haplotype.

The new P1 haplotype differs from the K haplotype in a single nucleotide at position 10,133 of the *cytb* gene. This haplotype was detected in only one locality (Suvi Do, Peshter plateau). Nevertheless, there was no variability within *cytb* sequences of *V. destructor* haplotypes within the K haplogroup on *A. mellifera* bees throughout Asia (Navajas et al. 2010). It is interesting that the locality, where we found the P1 haplotype, is inhabited by a particular *A. mellifera* ecotype (Stanimirovic et al. 2005a) that has specific behavioural and genetic characteristics (Cirkovic 2002; Stanimirovic et al. 2005b; Stevanovic 2007; Stevanovic et al. 2010; Muñoz et al. 2012). Analyses of mtDNA of the honey bees originating from this locality showed the presence of three different haplotypes, one of them being exclusive to this region (Muñoz et al. 2012). The existence of specific *A. mellifera* and *V. destructor* mtDNA haplotypes in the same region of Serbia is in line with the findings of Warrit et al. (2006) about biogeographical congruence between the *A. cerana* mitochondrial lineages and the *V. jacobsoni* mitochondrial haplotypes in Thailand.

Previously published data revealed an extreme lack of mtDNA polymorphism in *V. destructor* outside Asia. This was explained by genetic bottlenecks that occurred in Asia before and after mites shifted from the original *A. cerana* to the new host, *A. mellifera*

(Navajas et al. 2010). On the other hand, our data show the presence of conspicuous variability of *V. destructor* mtDNA in Serbia. However, the source of variability and the virulence of the haplotypes found remain to be examined.

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