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Genetic characterization of the mite *Varroa destructor* (Acari: Varroidae) collected from honey bees *Apis mellifera* (Hymenoptera, Apidae) in the State of Santa Catarina, Brazil

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ABSTRACT. The mite *Varroa destructor* is an ectoparasite that is considered a major pest for beekeeping with European honey bees. However, Africanized bee colonies are less threatened by this ectoparasite, because infestation levels remain low in these bees. The low reproductive ability of female mites of the Japanese biotype (J), introduced to Brazil early in the 1970s was initially considered the main factor for the lack of virulence of this parasite on Africanized bees. In other regions of the world where the Korean (K) biotype of this mite was introduced, there have been serious problems with *Varroa* due to the high reproductive potential of the mite. However, a significant increase in the reproductive rate of females of *Varroa* in Brazil has been recently demonstrated; the cause could be a change in the type of *Varroa* in the bee colonies. We evaluated the prevalence of haplotypes J and K in mite samples collected from the State of Santa Catarina and from the island of Fernando de Noronha in the State of Pernambuco. The analysis of the mitochondrial genome (PCR + RFLP) revealed haplotype K in all samples from Santa Catarina and haplotype J in all samples from Fernando de Noronha. The analysis of microsatellites (nuclear genome) in bees from Fernando de Noronha

showed only the specific alleles of haplotype J, while in bees from Santa Catarina, these alleles were found in only 2.8% of the samples. The high frequency of individuals with Korean genetic material is probably to the reason for the current high reproductive capacity of the mite *V. destructor* recorded in Santa Catarina.

Key words: Varroasis; Africanized bee; Haplotype; Microsatellites; Polymerase chain reaction + Restriction fragment length polymorphism

INTRODUCTION

Since the mite *Varroa destructor* (Andersen and Trueman, 2000) came into contact with the bees *Apis mellifera*, the varroasis pest has become one of the main problems of beekeeping worldwide (Botta et al., 2004). Besides the high rates of infestations reached by mite *V. destructor* in *A. mellifera* colonies, it is also considered to be a vector of various infectious diseases such as acute paralysis virus, the Kashmir virus and the deforming wing virus (Bakonyi et al., 2002; Chen et al., 2004; Tentcheva et al., 2006).

Although this parasite was introduced in Brazil at the beginning of the 1970s (Stort et al., 1981), the pest has maintained low levels of infestation in colonies of Africanized bees (Moretto and Mello Jr., 2001).

Several factors are considered to be relevant in the population dynamics of the mite *V. destructor*. Climate affects significantly the level of infestation attained by the mite (De Jong, 1984). In temperate climate conditions, high rates of infestation are observed after contact between mite and *A. mellifera*, unlike in tropical climate conditions where the rates of infestation are tolerable (Gonçalves, 1987; Moretto et al., 1991a; Silva et al., 1992; Moretto and Mello Jr., 2001).

According to Moretto et al. (1991b), the occurrence of defensive mechanisms, such as grooming and the hygienic behavior exhibited by Africanized bees, could be one of the causes for the low levels that this varroasis reaches these bees. However, several investigators support the hypothesis that the variation in the female reproductive capacity of the mite *V. destructor* on workers brood cells from different *A. mellifera* subspecies is the main factor for differences in the levels of infestation attained by the varroasis pest. In worker brood cells from African bees and their hybrids, the reproductive success of *Varroa* would be lower than with European bees; this would explain the low level of infestation caused by the mite *V. destructor* in Africanized bees in Brazil and other countries (Medina and Martin, 1999; Rosenkranz, 1999; Martin and Kryger, 2002; Calderon et al., 2003; Martin and Medina, 2004).

However, studies on the genetic variability of the mite *V. destructor* showed that the virulence of varroasis is also related to different *Varroa* types (Kraus and Hunt, 1995; Anderson and Fuchs, 1998; De Guzman and Rinderer, 1999; Warrit et al., 2004; Solignac et al., 2003, 2005). Anderson and Trueman (2000) based on the variability in the cytochrome C oxidative I (COI) gene of mitochondrial DNA (mtDNA) have determined the existence of Japanese and Korean haplotypes. The former is found where the pest reaches low infestation levels, as in South and Central America, while the Korean haplotype would be predominant where the *Varroa* is found with high infestation levels as in Europe.

The analysis of the nuclear genome through the use of microsatellites has also been used in the characterization of *Varroa* populations. Although Solignac et al. (2005) found low

variability in 20 loci of microsatellites analyzed in *V. destructor*, specific alleles for each type of *Varroa* haplotype were identified.

The low levels of mite infestation in Africanized bees in Brazil are attributed to, among other factors, the *Varroa* haplotype that colonized here - the Japanese haplotype (Anderson and Trueman, 2000). However, Carneiro et al. (2007) found that the reproduction rate of females of *V. destructor* in cells of young workers of Africanized bees in Brazil is currently almost twice as high when compared with the reproductive rate of twenty years ago. Thus, there is the possibility that the Japanese haplotype was replaced by the Korean one, causing an increase in the number of offspring left by the adult female mite in Brazil. Therefore, the present study sought to determine the occurrence of the type of *Varroa* (Japanese or Korean) that parasitizes apiaries in the State of Santa Catarina (southern Brazil) and to relate it to the disease situation currently found in the state.

MATERIAL AND METHODS

For the genetic characterization of nuclear and mitochondrial genomes, samples of adult females of *V. destructor* were collected from apiaries in the region of Blumenau, Joinville, São Joaquim, Mafra, and Caçador in the State of Santa Catarina and compared with mite samples collected from Fernando de Noronha, State of Pernambuco. The molecular analyses were conducted on 30 adult females, collected on offspring of workers and/or offspring of drones and/or on workers and adult drones from each of the localities. After collection, the mites were packed in 70% ethanol and then stored at -20°C.

The total DNA was individually extracted for each female of *V. destructor* using the method adapted by Anderson and Fuchs (1998). After washing in 70% ethanol, each mite was placed in a watch glass and with the aid of a dissection magnifying glass, it was dilacerated. It was transferred to a 1.5-mL tube containing 40 µL 2X lysis buffer (120 µg/mL proteinase K, 0.1 M KCl, 0.02 M Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.9% Tween 20, 0.9% NP40, and 0.02% gelatin). The tubes were first incubated at 65°C for 30 min, and then at 95-100°C for 10 min, with a final addition of 20 µL distilled H_2O. The extracted DNA was stored at -20°C.

For haplotype determination, the COI gene region of the mitochondrial genome of mite *V. destructor* was amplified by polymerase chain reaction (PCR) using the primers COXF [5'GG(A/G)GG(A/T)GA(C/T)CC(A/T)ATT(C/T)T(A/T)TATCAAC3'] and COXRa [5'GG(A/T)GACCTGT(A/TA(A/T)AATAGCAAATAC3'], described by Anderson and Fuchs (1998). PCR were carried out using 2-10 µL of the DNA extraction obtained by the method described above: 12.2 µL distilled deionized water, 1.8 µL PCR buffer, 1.8 µL 2 mM dNTPs, 0.55 µL 50 mM $MgCl_2$, 0.55 µL of each primer at 20 mM and 2.5 U Taq DNA polymerase. The amplification conditions used were: initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and 20 s of elongation at 64°C for 2 min. An extra elongation step at 64°C for 10 min was performed. Amplified products were separated by 0.8% agarose gel electrophoresis, stained with ethidium bromide and viewed with ultraviolet light. The identification of the Japanese and Korean haplotypes of the mite *V. destructor* was carried out based on digestion products with restriction enzymes, *Xho*I and *Sac*I, as described by Anderson and Fuchs (1998). Both haplotypes have the cleavage site for the enzyme *Xho*I, but only the Japonese pattern shows cleavage by the enzyme *Sac*I. The digestion products were examined on 1% agarose gels stained with ethidium bromide.

The analysis of the nuclear genome was done through the amplification of four loci of microsatellites (VD112, VD119, VD126, and VD152) using four pairs of primers designed by Solignac et al. (2003). Each PCR was carried out using: 3.8 µL distilled deionized water, 0.6 µL PCR buffer, 0.3 µL dNTPs, 2 mM each, 0.18 µL 50 mM $MgCl_2$, 0.12 µL of each primer at 20 µM, 1.5 µL of the DNA extracted and 1 U *Taq* DNA polymerase. The first step for the amplification consisted of denaturation for 3 min at 94°C. Next, the samples were submitted to 35 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 30 s of elongation at 72°C. Finally, an extra cycle of 10 min at 72°C was performed. The alleles of microsatellites were viewed on 12% polyacrylamide gels stained with silver nitrate.

RESULTS

PCR amplification of the mitochondrial genome COI region of the mite *V. destructor* generated a fragment of approximately 570 bp in all samples collected from Santa Catarina and Fernando de Noronha, PE. The digestions of these PCR products with the endonucleases *Xho*I and *Sac*I yielded polymorphic patterns between samples from Santa Catarina and Fernando de Noronha. PCR products digested with the enzyme *Xho*I produced fragments of 300 and 270 bp in all samples. However, when submitted to the enzyme *Sac*I, the 160 samples from Santa Catarina did not show the cleavage site, characteristic of *V. destructor* of the Korean haplotype, while the 30 samples from Fernando de Noronha generated fragments of 340 and 230 bp, exclusive of the Japanese haplotype (Figure 1).

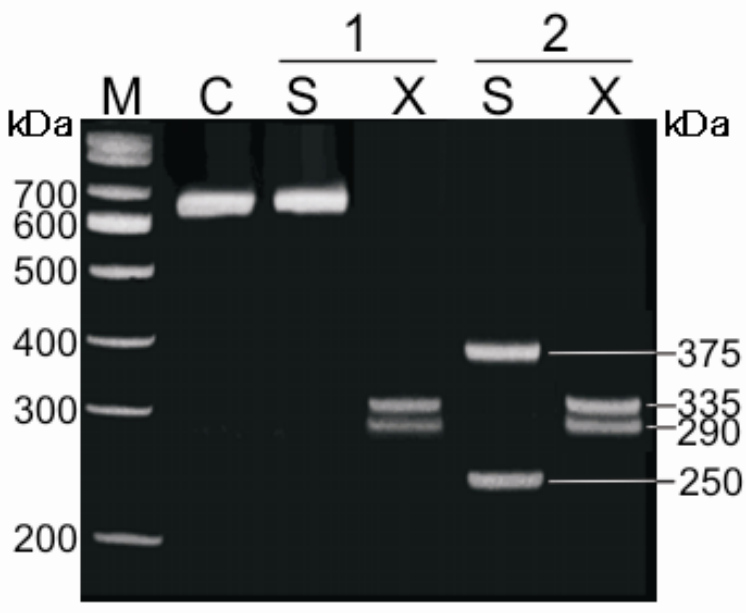

Figure 1. Restriction profiles of COI region of mtDNA from *Varroa destructor* collected in Santa Catarina (1) and Fernando de Noronha (2), where mtDNA was digested with endonucleases *Sac*I (S) and *Xho*I (X). *Lanes M* and *C* represent 100-bp DNA ladder and undigested amplified DNA, respectively.

Nuclear genome analyses of four microsatellite regions showed Japanese homozygous genotypes in all samples from Fernando de Noronha, and the Korean pattern was present in Santa Catarina samples, but exclusive only in the microsatellite VD112. In the other three microsatellite regions, the Japanese genotype was also found in 2.8% of the samples from this State. Microsatellite VD119 in all samples exhibited the Korean genotype type, except one mite from São Joaquim, which showed the homozygous Japanese genotype (Figure 2A). The polymorphism found in the VD126 microsatellite region was notable by the presence of two mites from Blumenau and one from the São Joaquim region, which showed the homozygous genotype of the Japanese biotype, while all other samples showed the typical Korean pattern (Figure 2B). In relation to the VD152 microsatellite region, a heterozygous mite for both genotypes was obtained in a sample from the Blumenau region (Figure 2C).

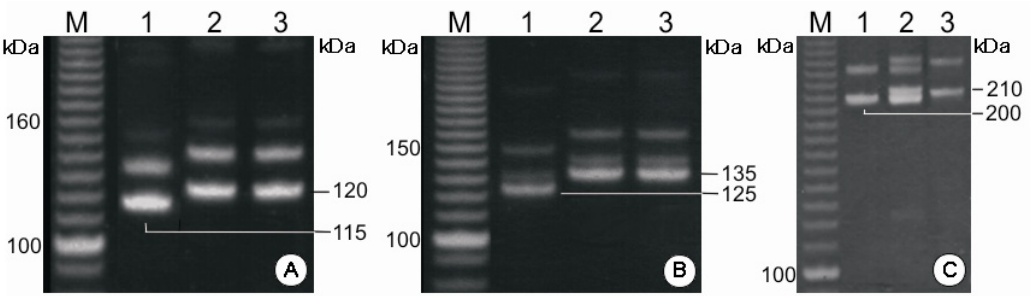

Figure 2. Restriction profiles of microsatellites VD119 (A), VD126 (B) and VD152 (C) from *Varroa destructor* collected in Santa Catarina (1 and 2) and Fernando de Noronha (3). M represents 10-bp DNA ladder.

DISCUSSION

The mite *V. destructor* is an invasive species that rapidly disseminates in *A. mellifera*, having a great impact around the world. However, in tropical and subtropical regions, particularly in South and Central America, the parasite has caused less damage to Africanized bees (De Jong and Soares, 1997). Some factors that are related to this low impact on bees, such as the mite tolerance of African bees and their hybrids (Medina and Martin, 1999), climatic conditions (Moretto et al., 1991a), and the different *Varroa* genotypes (De Guzman et al., 1998), seem to be important factors in the population dynamics of the mite *V. destructor*.

De Guzman et al. (1997) using RAPD markers differentiated two *Varroa* biotypes, namely the Russian genotype for the populations from the United States, Russia, Morocco, Germany, Italy, Spain, and Portugal and the Japanese genotype for populations from Japan, Brazil, and Puerto Rico. Anderson and Trueman (2000) using mtDNA markers also verified the biotypes as the Korean (= Russian) and Japanese haplotypes.

The results of the mitochondrial genome analysis obtained with samples from Santa Catarina corroborate those of Garrido et al. (2003) who found less than 2% of *Varroa* with the Japanese haplotype in samples collected in 1996 and 2001 in the cities of Ribeirão Preto (SP), Florianópolis (SC) and Estrela (RS). In all samples of this work collected in the State of Santa Catarina, the presence of the Japanese haplotype was not observed either.

The presence of the Japanese pattern in all samples from Fernando de Noronha archipelago could be attributed to the geographical isolation of the bee colonies from continental Brazil. This fact supports the hypothesis that the Japanese biotype of *Varroa* would have been the first one to enter the country through the imported queens from Japan to Paraguay and then to Brazil (De Jong and Gonçalves, 1981). The *Varroa* mite introduction in Fernando de Noronha could have occurred since 1984, when some *A. mellifera* from continent were introduced at Archipelago (Guerra Jr. et al., 2000). The creation of the Fernando de Noronha National Marine Park, in 1988, prohibited the introduction of any species of animal, this could explain the current absence of the Korean haplotype on the island, which was introduced in Brazil later than the Japanese haplotype. The fact that Fernando de Noronha has a population of mites that was brought from the continent more than 20 years ago supports the hypothesis that the Japanese haplotype was the first type of *Varroa* introduced in Brazil.

Data from microsatellites showed low variability in the four loci studied. Solignac et al. (2005) also found heterozygosity to be less than 1.3% in 11 regions of microsatellites analyzed from 45 populations of mites from 17 countries. According to Tsutsui et al. (2000), low variability is quite common in invasive species because of the frequent occurrence of the founder effect. Another important factor in the genetic structure of mite populations is their reproductive system. The determination of sex is based on the haploid-diploid (De Ruijter and Papas, 1983 *apud* Martin et al., 1997) and pseudo-arrhenotoky systems (Martin et al., 1997). Thus, a founder adult diploid female enters a bee offspring cell to reproduce and lay haploid eggs, which will generate males. The pseudo-arrhenotoky condition is characterized by the set of chromosomes that comprise the non-fertilized ovule to be solely of maternal origin. The ovipositions that originate females are composed of fertilized ovules (diploid). Since the fertilization of daughter females occurs when the cell still has an operculum, it usually occurs between siblings (Donze et al., 1996). Therefore, this high inbreeding rate should be responsible for the low genetic variability found in the mite *V. destructor*.

Although the occurrence of specific alleles was low, samples from the State of Santa Catarina could explain the possible presence of Japanese biotype mites in the past, which was changed by the Korean genotype/haplotype. The Japanese biotype, characterized by lower virulence when compared to the Korean biotype (Delfinado-Baker, 1988), was a factor suggested by Anderson and Trueman (2000) for the low virulence of the mite in Brazil. The possible alteration of the mite biotype in Brazil is also suggested by Carneiro et al. (2007) who found an increase in the reproductive ability of the mite when compared with data from the 1980s. The fertility results showed that in that period, possibly when the mite populations were formed by Japanese biotypes, each founder female produced an average of 1.3 deutonymphs, while currently this average is 1.7 deutonymphs per *Varroa*. This significant increase in descendants is similar to that found in European bees from the United Kingdom (Medina and Martin, 1999), where the Korean pattern has been determined since 1997 (Anderson and Trueman, 2000).

However, despite this possible change in genotype/haplotype of *Varroa* in the State of Santa Catarina, the level of infestation of the parasite in adult bees is still considered low, approximately 4% (Carneiro FE, personal communication). This value is similar to that found in the early 1990s (Moretto et al., 1991a). Several factors have contributed to the increased tolerance of *A. mellifera* bee (Africanized) to the parasite. The hygienic behavior and grooming have been reported as key mechanisms of resistance to the mite (Boecking and Spivak, 1999). Moretto et al. (1993) showed that Africanized bees show 38% cleaning capacity, while Italian

bees only 5%. Moretto et al. (2006) found that the daily rate of cells without operculum was 3.5 times greater in beehives infested with *Varroa* compared with non-infested beehives. This shows that Africanized bees have the ability to recognize and remove offspring naturally parasitized with the mite. In the grooming activity, Junkes et al. (2007) found a significant increase in mites on the bottom of the beehive as the amount of offspring of workers decreases in the bee colonies. This result suggests that the activity increased as the concentration of mites in the population of adult bees also increased, resulting in a higher mortality rate of mites.

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