Original Article

CHARACTERIZATION OF *VARROA DESTRUCTOR* MITES IN CUBA USING MITOCHONDRIAL AND NUCLEAR MARKERS

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Abstract

***Varroa destructor* has been present in Cuba since 1996, but without the use of acaricidal infestation rates remain at very low levels. The presence of Korean haplotype mites was described in 2007, but there is no information regarding the introgression of the less virulent Japanese haplotype that could account for a low pathogenicity of the mite. In this research, we carried out molecular characterization of Cuban Varroa mites through mitochondrial DNA and hypervariable nuclear loci. We applied an alternative RFLP technique and found that all the analyzed samples corresponded to Korean haplotypes. We analyzed the three STRs loci VD112, VD114 and VD016, previously described as highly variable and found new alleles in all of them, with an absolute allele size very different to those reported worldwide. We also detected genic and genotypic differentiation between samples from two nearby locations (P=0.08). We also tested a new RFLP method for mite haplotype discrimination with an intra-reaction positive control of digestion.**

Keywords: DNA mitochondrial, haplotype, microsatellites, STR

INTRODUCTION

The *Varroa destructor* mite has been considered one of the main pests of honeybees *Apis mellifera* for decades. Infestation by this mite affects the individual bee and the colony (Rosenkranz, Aumeier, & Ziegelmann, 2010) and extraoral digestion of fat body affects immune functions, pesticide eliminations, overwinter survival and several essential processes in healthy bees (Ramsey et al., 2019). In addition, the mite acts as a vector for numerous viruses that, before the expansion of *V. destructor*, were considered a minor problem in beekeeping (Boecking & Genersch, 2008; Martin et al., 2012; Yue & Genersch, 2005). At the colony level, low levels of infestation by *V. destructor* give rise to undetectable symptoms, but an increase in the number of mites leads to a progressive decrease in the number of bees (Fries et al., 2003).

Eighteen haplotypes of *V. destructor* were described by Navajas et al. (2010) based on 2700 base pairs of the mitochondrial genome, and recently a few more were described by Traynor et al. (2020). From this wide variety, only two haplogroups successfully parasitize *A. mellifera.* The Japanese (J) haplotype is geographically more restricted and has been described in some colonies from Japan, Thailand, and South America, the latter specifically on the island of Fernando de Noronha in Brazil (Anderson & Trueman, 2000; Guerra Jr et al., 2010; Strapazzon et al., 2009). Some recent studies describe Japanese haplogroups in Europe and North America but on a limited scale (see Utzeri et al., 2009). Conversely, the Korean (K) haplotype is the most widespread worldwide, being present in Europe, the Middle East, Africa, Asia, the Americas, Australia and New Zealand (Anderson & Trueman, 2000; Muñoz et al., 2008). Moreover, the K haplotype is frequently described as more pathogenic on the base of its larger reproductive capacity (de Guzman & Rinderer, 1999; de Mattos, De Jong, &

Soares, 2016).

In Cuba, the first report of the *V. destructor* (previously identified as *V. jacobsoni*) dates from 1996 but some evidence suggests that the mite may have been introduced to the country two years earlier (Demedio, 2001). In 1997 the mite was considered responsible for the loss of around 8000 honeybee colonies (Verde & Demedio, 1998) expanding to seven provinces of the country by the following year. Since the introduction of *V. destructor to* Cuba, several studies have been conducted to identify the use and effectiveness of such pest control methods as flumetrin (Bayvarol®) and organic acid (API LIFE Var®) (Domínguez, 2004; García, 2004; Pérez Morfi, 2014). Acaricides have not been used in the Cuba for more than over twenty years. At present, mite control is based on the selection of Varroa-tolerant bees taking advantage of natural defensive mechanisms and physiological characteristics of colonies surviving varroasis, especially Varroa Sensitive Hygiene (VSH) (Sanabria, 2007). To date, sustained levels of mite infestation rate have been maintained in Cuba at only 2.2-3.6% (Pérez Morfi, 2014).

An initial morphometric description of *V. destructor* in Cuba was conducted in 2001 (Demedio, 2001), but this phenotypic characterization did not allow for a proper identification of the haplotypes present in the country. The first molecular characterization of Cuban mites in 2010 described the presence of haplotype K in seven mites of unknown geographical origin by the traditional PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique using a mitochondrial COI gene (Guerra Jr et al., 2010). However, the tolerance/resistance of Cuban honeybees to varroasis suggests the presence of such low pathogenic strains of *V. destructor* as the J haplotype or a still unreported K variant. Although the haplotype K, currently distributed worldwide, is also clearly present in Cuba, the existence of the less virulent Japanese variant in the country is uncertain. Therefore, the existence of the J haplotype may have left a legacy of less virulent mites, naturally selected to coexist better with honeybees. Traces of such mites could be found in such hypervariable nuclear markers as STRs (Short Tandem Repeat) or microsatellites, of biparental inheritance. The aim of the present study is to characterize the *V. destructor* present in Cuba, using the traditional PCR-RFLP technique for the study of mitochondrial genome sequences and STRs markers.

MATERIAL AND METHODS

Sample collection

Adult *V. destructor* mites were collected from eight provinces located throughout Cuba. Samples from the western region were collected in Pinar del Río, La Habana, Mayabeque, and Matanzas during April 2018 and subsequently numbered from 1 to 4, respectively. The eastern provinces Las Tunas, Holguín, Santiago de Cuba and Guantánamo were sampled in May of the same year and subsequently numbered from 5 to 8, respectively (Fig. 1). Four colonies per province were sampled. All the mites were collected in hybrid colonies of M and C lineages (Yadró unpublished). Mites were preserved in ethanol 70% and stored at -20°C until analysis. Mitochondrial PCR-RFLP analyses were conducted on 320 adult females, forty from each province. STR genotyping was carried out in samples from La Habana and Mayabeque.

Molecular analyses

The total DNA was individually extracted for each mite through the conventional method described by Miller, Dykes, and Polesky (1988) with modifications. Subsequently, the extracted DNA was purified using Wizard™ Minipreps DNA Purification System (Promega) to clean impurities and salts that inhibit the PCR reaction. Haplotype determination was carried out by two alternative PCR-RFLP methods on cytochrome C oxidase 1 (COI) gene of the mitochondrial genome. For both methods, amplifications were carried out using GoTaq® Hot Start Green Master Mix (Promega). The reaction mix contained 7.5 µL of GoTaq® Hot Start Green Master Mix 2X, 1.5 µL of each primer (10 µM) and 4.5 µL of DNA template for a final volume of 15 µL.

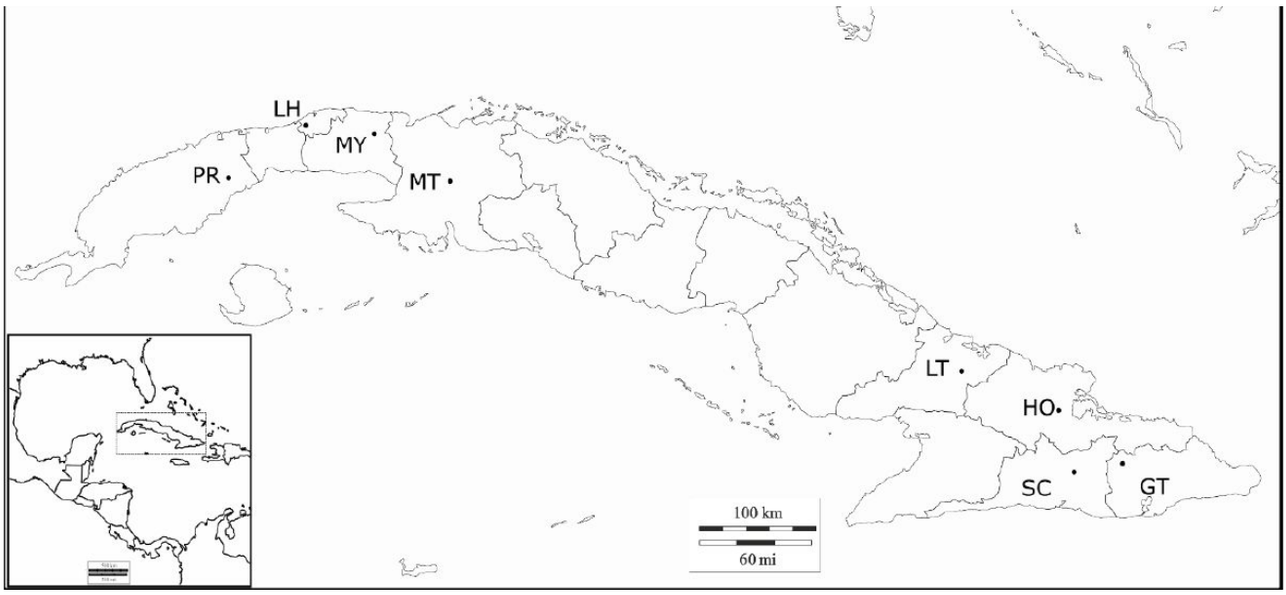

Fig. 1. Map of sampled locations. PR: Pinar del Rio, LH: La Habana, MY: Mayabeque, MT: Matanzas, LT: Las Tunas, HO: Holguín, SC: Santiago de Cuba, GT: Guantánamo. Black triangles represent localization on the sampled apiaries.

The first method amplifies a region of 570 bp (Navajas et al., 2002) using the primers COXF and COXRa. The amplicons were digested with SacI which generate two restriction fragments for J haplotype (230 and 340 bp) but produce no cut in K haplotype because of the lack of restriction site (Anderson & Trueman, 2000). The amplification conditions used were initial denaturing at 94°C for 5 min, thirty-five cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and elongation at 72°C for 1 min and ends with a final elongation of 72°C for 10 min. Restriction assay was achieved using SacI (Thermo Scientific) mixing 10 µL of each sample with 16 µL of Nuclease Free Water (Promega), 2 µL of 10X Buffer for SacI and 2 µL on SacI. Samples were incubated at 37°C for 2h. Due to the difficulty of having a positive control for the J haplotype, we decided to use an alternative PCR-RFLP method (Mendoza unpublished) with internal control of digestion, in which both haplotypes had restriction sites. This new method amplifies a 470 bp fragment between positions 276 and 736 of COI using primers (VD-CO206F: 5´ACCAGATATAGCTTTTCCACG3´, VD-CO650R: 5´AAATATAAACTTCTGGGTGTCC3´). The Amplified region digested with EcoNI generates three restriction fragments for J haplotype (68, 129, and 273 bp) and two restriction fragments for K haplotype (129 and 341 bp). The amplification conditions used were initial denaturing at 94°C for 5 min, thirty cycles of denaturation at 94°C for 45 s, annealing at 47°C for 15 min and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. In this case, the restriction was carried out mixing 8 µL amplification products, 1µL of NE Buffer 4 (New England Biolabs) and 1µL of EcoNI (New England Biolabs). Digested products were electrophoresed in 2% agarose gel for approximately 30 min at 120V and then visualized through ethidium bromide staining in a UV transilluminator.

STRs loci VD016, VD112, and VD114 described by Solignac et al. (2003) were amplified and genotyped. These loci were selected because they were described as polymorphic and their allele variation associated with the geographical origin of *Varroa* strains (J or K haplotypes) (Solignac et al., 2005). Multiplex amplification of the three STRs was carried out using GoTaq® Hot Start Green Master Mix from Promega. Reaction mix contained 10 µL of GoTaq® Hot Start Green Master Mix 2X, 0.35 µL of each primer (40 µM), 7.2 µL of DNA template for a final volume of 20 µL. PCR conditions were initial denaturing at 94°C for 3 min, thirty-five cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s and a final elongation at 72°C for 10 min.

The PCR products were electrophoresed in 8-mm-thick denaturing polyacrylamide gels (acrylamide:bisacrylamide 19:1 and 7% urea) for approximately 4h at 1,000-1,200V and visualized through silver staining modified from Sanguinetti, Dias & Simpson (1994). Individual genotypes were independently determined by two observers and absolute allele size estimated through comparing with a 10 bp standard size (GIBCO BRL).

Statistical analyses

STR data was analyzed for the presence of null alleles, large allele dropout and scoring errors using MICROCHECKER v.2.2.3 (Van Oosterhout et al., 2004). We assessed departure from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and exact tests for genic and genotypic population differentiation for each locus independently and all loci together using GenePop (Raymond & Rousset, 1995), excluding monomorphic locus VD016. Allelic frequencies and heterozygosis were performed in Genalex (Peakall & Smouse, 2006). Exact test overall and pairwise Fst estimates were calculated in Genalex. To evaluate the partitioning of genetic variation within and among individuals and between mite populations we used a one-way analysis of molecular variance (AMOVA) in Genalex.

RESULTS

The COI region of the mite *V. destructor* was successfully amplified in all samples. The digestion of these amplicons with the endonucleases SacI and EcoNI showed in both cases a characteristic pattern of K haplotype. All samples were not digested by the enzyme SacI, and amplicons digested with EcoNI generated fragments approximately of 150 and 330 bp (Fig. 2). The size of digested bands corresponds to *in silico* simulations and to the expected size. This new variant will be very helpful so there is no need to use an external positive control for digestion. STRs loci were successfully amplified and a summary of the results is in Tab. 1. All analyzed loci have alleles with an absolute size much larger than those previously reported for the species (Solignac et al., 2003, 2005). The differences in size are greater than would be expected due to the different analytical and interpretation methods. The locus VD016 was monomorphic, with a single allele of 316 bp. Loci VD112 and VD114 were polymorphic, with four and three alleles scored respectively. In the case of VD112, only one allele represented 77% of detected alleles. For VD114, similarly only one allele represented the 91.3% in frequency.
No evidence of null alleles or of linkage disequilibrium was detected between polymorphic loci

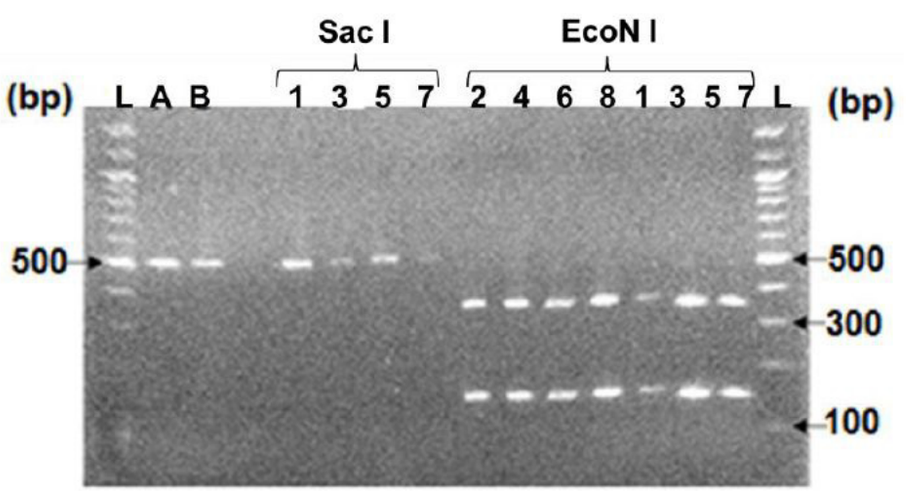

Fig. 2. Restriction profiles of COI region of *Varroa destructor* mtDNA. Eight provinces of Cuba were sampled and displayed numbered from 1 to 8. 1: PR, 2: LH, 3: MY, 4: MT, 5: LT, 6: HO, 7: SC and 8:GT. The mtDNA was digested with endonucleases SacI and EcoNI. Lane L: 100-bp DNA ladder, Lane A: Undigested amplicons (amplified with COXF and COXRa primers) and Lane B: Undigested amplicons (amplified with VD-CO206F and VD-CO206R).

Table 1.

Microsatellites absolute sizes and frequencies of alleles found in Cuba. He and Ho are expected and observed heterocigocity, respectively

	Allele size (bp)		Localities	
Locus	sequenced *	this study	Mayabeque	La Habana
			N=12	**N=12**
VD112	142	158	0.04	0.04
		160	0.00	0.08
		168	0.88	0.58
		178	0.08	0.29
Ho			0.25	0.67
He			0.23	0.57
			N=11	**N=12**
VD114	217	246	0.09	0.08
		250	0.91	0.92
Ho			0.00	0.00
He			0.17	0.15
VD016	268	316	**N=12**	**N=12**
			1	1
Ho			0	0
He			0	0

*alleles reported by Solignac et al. 2003.

(p=1), but statistical significant departures from HWE was detected in both loci in all samples and by population ($p < 0.05$). Exact tests for genic and genotypic population differentiation were neither significant for all loci (P=0.27) nor by loci but almost marginal for VD112 (p=0.08, both genic and genotypic). Furthermore, the overall and pairwise Fst values for locus VD112 indicate a differentiation between La Habana and Mayabeque populations (Fst 0.11). However, AMOVA calculations given by GenAlex show that a higher percentage of variation occurs among individuals (96%) and within individuals (4%), but no variation was detected among populations.

DISCUSSION

This study presents the first molecular characterization of Cuban *Varroa destructor* mites through the analysis of mitochondrial and nuclear markers, which significantly contributes to the information that the scientific community has. This country, isolated due to its insular condition with mite-tolerant honeybees, and with an official ban on importing honeybees, represents a unique opportunity to study the natural variation of these mites, and ultimately, the parasite host-relationship.

The proposed PCR-RFLP fulfilled our expectations, and we successfully discriminated J/K haplogroups in the absence of Japanese haplogroup control. The presence of K haplotype is not surprising as it is common throughout the world (Anderson & Trueman, 2000; Rosenkranz, Aumeier, & Ziegelmann, 2010). This result is in concordance with previous studies in which the K haplotype was detected in seven samples of Cuban mites (Guerra Jr et al. 2010). This haplotype is described as the most pathogenic. Following the first detection of *V. destructor* in Cuba, there was a loss of numerous colonies throughout the country and a strong decrease

in honey production.

However, today varroasis is not a problem in Cuba possibly due to an increased resistance of honeybee populations. For example, a greater expression of Varroa Sensitive Hygiene (VSH) is possibly favored by the natural selection and the intentional breeding of the bees that survived this pathogen without the use of chemical treatments. The program of selection, management, and improvement of Cuban honeybees carried out since 2013 may have contributed to the increased resistance of the populations of Cuban honeybee through the selection and reproduction of the most hygienic honeybees. This program is based on the selection and breeding of colonies with the highest production rates but also infestation rates and hygienic behavior.

More interesting results were obtained from STR loci genotyped in order to find traces of the J haplotype in Cuban mites. The three genotyped loci were selected for this study because they were polymorphic and their allelic variation was associated with the different mitochondrial haplotypes (Solignac et al., 2005). The genotyped loci VD112 and VD114 turned out to be polymorphic while VD016 was monomorphic. The difference in the third locus is not striking and can be explained through drift allele fixation. The absolute size of found alleles differed in 16, 30 and almost 50 bp for VD112, VD114 and VD016 loci, respectively, when compared to the absolute size previously reported for those loci by Solignac et al. (2003). Recently, Dieteman et al. (2019) analyzed *V. destructor* from Thailand and found alleles ranging from 133 to 145bp for the VD112 locus. Although the difference of at least 10bp in absolute size is large enough for methodological differences, we cannot rule out that part of the range of variation is shared between both studies (this study ranged from 158 to 178bp). In any case, we consider that at least the largest alleles that we found in this study are new to the species. These findings suggest that the Cuban population has considerably differed from those studied so far worldwide, presenting particular alleles, which are reported for the first time in this study. This reflects the almost total isolation of the island from the mainland, consistent with the results of the molecular characterization of honeybees (Yadró et al., 2020), and as will be explained below, maintaining the natural variation of the mites.

In addition to finding new alleles for VD112, its variation is greater than previously reported for some global studies. Solignac et al. (2005) reported only three alleles for this locus worldwide, but for the two genotyped Cuban populations, La Habana and Mayabeque, we have found four alleles. Similar results were reported by Dietemann et al. (2019) in Thailand, who found four alleles. There is extensive evidence that *V. destructor* experiences high selective pressures and rapidly evolves in response to pesticide treatments (reviewed by Eliash & Mikheyev, 2020). Under these conditions, the selection of resistant mites reduces the genetic variation in its populations, a process known as "selective sweep" in population genetics (Smith & Haigh, 1974). Many studies that reported low genetic variation in *V. destructor* come from places with mite-susceptible colonies that need to be treated to survive. In Cuba, on the contrary, there are mite-resistant colonies that survive well with no acaricidal treatments, and therefore *V. destructor* populations can be evolving in a co-evolutionary arms race, to the selection pressure induced by their honeybee host. Beaurepaire et al. (2019) noted the adaptive ability of *V. destructor* as a possible factor in a host-parasite arms race, which can be registered in less than ten years. Then, the higher hygienic behavior of the Cuban honeybees could be the main driver of this greater variation.

In addition, we observed a considerable difference, both at a genic and genotypic level, between the two populations studied, which are located at a distance of around 70 km from each other and with no geographical barriers between them. This variation is not reflected in the differentiation tests, possibly due to the low sample size (N=12 in each population). The heterozygosity of the two populations also differs, which could reflect differences in the population sizes between localities, if we assume that STR used

are neutral loci. Under neutrality, the balance between genetic drift and neutral mutation would result in larger populations presenting a greater heterozygosis than those of smaller size. In this case, the differences observed might be a consequence of a lower density of colonies in La Habana, a more anthropized landscape, than in Mayabeque. Additionally, beekeepers in such countryside areas as Mayabeque tend to move colonies following the flowering periods with movements of the colonies beyond the restrictive province borders. The sampled apiary in Mayabeque moves its colonies no more than 10 Km. In contrast, samples in La Habana were collected in the experimental apiary of Centro de Investigaciones Apícolas (CIAPI) where apiaries are not moved around.

In summary, the results show that the current *Varroa* populations in Cuba have the Korean mitochondrial haplotype, and there is no evidence of the presence of the J haplotype as has been described in most of the world. The genotypes in the three STR loci studied do not allow us to conclude anything about the geographical origin of the mites. Interestingly, the presence of exclusive alleles in the three microsatellite loci is striking, especially for VD112, which reflect differentiation in isolation and high population sizes in the absence of acaricidal treatments.

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