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5 **Original article - Parasitology Research**
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9 # Genetic variation and heteroplasmy of *Varroa destructor* inferred from
10 # ND4 mtDNA sequences
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25
26 **Short title**: Genetic variability of *Varroa destructor*

27 **Abstract**

28 *Varroa destructor,* a parasitic mite of the Western honey bee *Apis mellifera L*., is a serious
29 threat to colonies and beekeeping worldwide. Population genetics of the mite has provided
30 information of two mitochondrial haplotypes infecting honey bee colonies, named K (Korea)
31 and J (Japan). In the American continent, the K haplotype was most frequently found. The aim
32 of this research was to study the genetic diversity of *V. destructor* populations from the major
33 beekeeping region of Argentina, previously unexplored. Phoretic mites were collected from
34 managed *A. mellifera* colonies in ten localities, and four mtDNA regions (*cox1*, *nad4*, *nad4L,*
35 and *nad5*) were analyzed. Based on *cox1* sequence, the exclusive presence of K haplotype was
36 detected. Two sub-haplotypes (KArg-N1 and KArg-N2) were identified from a variation in
37 *nad4* sequence. The frequency of these sub-haplotypes significantly correlates with the
38 geographic latitude. The occurrence of site heteroplasmy was also evidenced for this gene. The
39 *nad4* mtDNA marker appears to be a sensitive marker to detect genetic variability in mite
40 populations. The site heteroplasmy emerges as a phenomenon that could be relatively frequent
41 in *V. destructor*.

42

43 **Keywords:**

44 Mites; genetic variability; haplotypes; mtDNA heteroplasmy; molecular markers; *Apis mellifera*

45

46 **Introduction**

47

48 *Varroa destructor* Anderson and Trueman (Acari: Varroidae) is an ectoparasite considered the
49 main pathogen of the western honey bee (*Apis mellifera* L) colonies worldwide (Neumann and
50 Carreck 2010; Rosenkranz et al. 2010; VanEngelsdorp et al. 2011; Le Conte and Mondet 2017).
51 This species, initially named *Varroa jacobsoni*, is native to Southeast Asia. During the first part
52 of the twentieth century, this mite shifted from its natural host, the Asian honey bee *Apis cerana*
53 Fabricius, to *A. mellifera* and thereafter rapidly spread all over Europe, North America, South

54 America, Africa, and the Asia-Pacific region (Matheson 1995; Oldroyd 1999; Anderson and
55 Trueman 2000).
56 *V. destructor* was first introduced in South America (Paraguay) when beekeepers acquired *A.*
57 *mellifera* queens from Japan (De Jong et al. 1982), leading to a subsequent invasion of the
58 Argentinean territory (Montiel and Piola 1976). The mite was first detected in Argentina in
59 1976, in the northern province of Formosa, next to the Paraguayan border (Maggi et al. 2012).
60 Since its introduction, the mite has dispersed all over Argentinean colonies, producing a
61 negative impact on local honey bee populations and currently representing the major threat to
62 the beekeeping industry (Eguaras and Ruffinengo 2006; Vandame and Palacio 2010; reviewed
63 by Maggi et al. 2016).
64 The genetic variability of *V. destructor* populations has been assessed at a global scale
65 by using mitochondrial and nuclear markers (Anderson and Trueman 2000; Evans 2000;
66 Solignac et al. 2005; Navajas et al. 2010; Dynes et al 2016; Dietemann et al. 2019). Based on
67 mitochondrial DNA markers, multiple haplotypes have been described for *V. destructor*
68 populations infesting *Apis cerana* and/or *Apis mellifera* (Anderson and Trueman 2000; Zhou et
69 al. 2004, Navajas et al., 2010). However, only two of them, the Korean (K) and the Japanese (J)
70 haplotypes, were reported to have successfully reproduced in *A. mellifera* colonies (Anderson
71 and Trueman 2000). The J haplotype was detected in Japan, Thailand, and some regions of
72 Brazil, whereas the K haplotype was found globally distributed (de Guzman et al. 1999;
73 Anderson 2000; Anderson and Trueman 2000; Garrido et al. 2003; Solignac et al. 2005; Guerra
74 et al. 2010, Kelomey et al. 2017; Dietemann et al. 2019). Specifically, for South America,
75 previous studies detected the presence of the K haplotype in Venezuela, Chile, Uruguay,
76 Colombia, Brazil and Argentina (Garrido et al. 2003; Guerra et al. 2010). Subsequently, Maggi
77 et al. (2012) analyzed *cox1* mt sequences and inferred the exclusive presence of the K haplotype
78 and a low genetic variability in mite populations of central-eastern and southern regions of
79 Argentina.
80 Several studies on the genetic variation of *V. destructor* populations have used the
81 aforementioned mtDNA marker (*cox1* gene). This marker has demonstrated to be useful to

82 identify mites at the species level and to define particular populations ('haplogroups') (Koeniger
83 et al. 2002; Zhou et al. 2004; Solignac et al. 2005; Strapazzon et al. 2009; Muñoz et al. 2008;
84 Navajas et al. 2010). As variation in this gene region is less than 2% among mites of the same
85 species (Anderson and Trueman 2000), the use of this marker alone may not be sensitive
86 enough to detect inter-population variation. Navajas et al. (2010) found that the combined use of
87 *cox1* and other mitochondrial DNA markers (*atp6*, *cox3,* and *cytb*) allows the detection of
88 genetic variation both within and between different *V. destructor* populations worldwide (see
89 also Elbeaino et al. 2016 and Farjamfar et al. 2018). In fact, based on these markers, the
90 occurrence of new sub-haplotypes within the K haplotype of *V. destructor* has been described
91 (Navajas et al. 2010; Gajic et al. 2013). Another mtDNA gene, encoding the NADH
92 dehydrogenase, has proven to be highly polymorphic in a large number of insect species
93 (Michel et al. 2006; Meraner et al. 2008; Fernández et al. 2013) and arachnids (e.g. Masta 2000;
94 Li et al. 2017; Liu et al. 2018). Although this mt marker has been successfully applied to
95 population genetic studies on acari (e.g. Li et al. 2019), its use has not been previously reported
96 in genetic studies of *V. destructor* populations.
97 Preliminary results obtained through PCR and sequencing suggest that size
98 heteroplasmy (mtDNA molecules within the same cell or individual differing in nucleotide
99 sequence size) is present in *V. destructor* (Navajas et al. 2002). In a recent study on Serbian
100 populations of the mite, site heteroplasmy (mtDNA molecules within the same cell or individual
101 differing in the nucleotide composition) was evidenced by the presence of double peaks in
102 punctual positions of *cox1* and *cytb* gene sequences (Gajic et al. 2016; 2019).
103 The aims of the present study were to analyze the genetic variability of *V. destructor*
104 populations belonging to the main apicultural area of Argentina by using previously unexplored
105 mtDNA markers (three subunits of the gene encoding the NADH dehydrogenase) and to
106 evaluate at nucleotide level the presence of heteroplasmy in these populations. This study sheds
107 light on how genetic variability of *V. destructor* is distributed within and between
108 geographically distant populations of Argentina and contributes to developing new markers that
109 may be applied to future studies on worldwide mite populations.

110

111 **Material and methods**

112

113 **Sample collection**

114

115 Managed colonies of *A. mellifera L.* from apiaries in ten localities of Argentina were sampled.
116 These localities, selected due to their importance in apicultural activities, are situated in
117 different provinces of the country representative of subtropical and temperate climates (Table 1,
118 Fig. 1). In all cases, mites were collected alive from the bodies of adult worker bees (phoretic
119 mites) and stored in labeled plastic flasks containing ethanol 70% (v/v). At least 20% of the
120 total honey bee colonies were randomly sampled from each apiary (one apiary for each
121 locality). The number of sampled colonies per apiary ranged from three to ten, according to the
122 size of the apiary. Almost five individual mites were randomly collected from each colony.
123 When a sampled colony was found to have a low mite load, all available individuals were
124 analyzed (≥3). A total of 50 honey bee colonies were sampled, and 182 mites were collected.
125 One worker honey bee from each sampled colony was preserved for genetic characterization
126 (Table 1).

127

128 **DNA extraction and PCR amplification**

129

130 Total DNA was extracted from single female mites according to the method proposed by
131 Baruffi et al. (1995) with the following slight modifications associated with the size of the mite:
132 (i) all volumes were reduced to half; (ii) centrifugation times were increased; (iii) final elution
133 was reduced to 10 µL of TE buffer (Tris base 10mM; EDTA 1mM). The DNA samples were
134 quantified using Nanodrop 1000 (Thermo Scientific) and stored in a freezer at -20 °C.
135 Four fragments of the mtDNA, including a segment of the cytochrome oxidase
136 subunit I (*cox1*) gene and three regions corresponding to the NADH dehydrogenase enzyme
137 gene [subunit 4 (*nad4*), subunit 4L (*nad4L*), and subunit 5 (*nad5*)], were amplified by

138 polymerase chain reaction (PCR) from each *V. destructor* DNA sample. In particular, *cox1* gene
139 fragment was amplified using a forward primer (10KbCOIF1, Navajas et al. 2010) and a reverse
140 primer designed from the reference sequences (GenBank accession number AY163547.1 from
141 Evans and Lopez 2002; and AJ493124.2 from Navajas et al. 2002). *Nad* regions were amplified
142 using newly designed primers (Table S1). These primers were designed considering the
143 presence of nucleotide changes (potential polymorphic regions) between the available reference
144 sequences from the *V. destructor* mtDNA genome. An initial set of 30 randomly selected DNA
145 samples (3 individuals per population) was analyzed for all the aforementioned mtDNA markers
146 in order to explore the presence of polymorphisms in *V. destructor* Argentinean populations.
147 The DNA set was expanded for mtDNA regions showing nucleotide variation, considering a
148 representative number of DNA samples for each population.
149 PCR amplifications were performed in a final volume of 20 µL. The reaction mix was
150 prepared with 50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 2% Sucrose and 0.25 mg/ml BSA, 200
151 µM of dNTP, 0.5 µM of each oligonucleotide, 0.04 U/µL Taq polymerase (Inbio HighWay,
152 Tandil, Argentina), 1 µL of the template DNA, and distilled water (Invitrogen, Ultrapure). PCR
153 cycling conditions were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles
154 of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and an extension at 72 °C for 80 s.
155 An extra elongation step at 72 °C for 10 min was also applied. All PCRs were performed on an
156 Applied Biosystems Veriti ™ Thermal Cycler. PCR products were electrophoresed in 1.5% w/v
157 agarose gels in 0.5X TBE, stained with 1 µg/mL of ethidium bromide, and visualized under UV
158 light (Syngene).
159 Mitochondrial haplotypes of worker honey bees for each analyzed colony were assessed
160 following Pinto et al. (2003).
161
162 **Sequence analyses**
163
164 PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following the
165 manufacturer's protocol. The quality and quantity of the purified samples were evaluated using

166 Nanodrop 1000 (Thermo Scientific). The purified PCR samples were sequenced using the
167 forward and reverse primers in an automatic capillary sequencer (Hitachi ABI 3130, Applied
168 Biosystem) at the DNA Sequencing and Genotyping Service, SIGYSA – CICVyA (INTA,
169 Castelar, Buenos Aires, Argentina). All obtained sequences were compared to the GenBank
170 (NCBI) nucleotide database using BLASTN (Altschul et al. 1990). Sequences were analyzed
171 using BioEdit (Hall1999). Alignments were performed using the reference sequences from the
172 complete mitochondrial DNA of *V. destructor* (Evans and Lopez 2002; Navajas et al. 2002),
173 and the individual sequences of the K haplotype obtained in previous studies (e.g. Anderson and
174 Trueman 2000; Navajas et al. 2010).
175
176 **Heteroplasmy verification**
177
178 *Nad4-nad4L* PCR products showing double peaks in the chromatograms were cloned using the
179 pGEM-T Easy kit (Promega) according to the manufacturer's instructions. We selected and
180 analyzed at least two samples from *nad4-nad4L* PCR fragments (Table S1). Ten clones of each
181 sample were sequenced using SP6-T7 primers. The obtained sequences were analyzed as
182 mentioned above. Only sequences from individuals exhibiting a double peak at 8694 nucleotide
183 position (the most frequently detected change) were obtained. Other identified double peaks
184 were considered potentially heteroplasmic sites because it was not possible to obtain good
185 quality sequences from the cloned fragments. See details in Table 2.
186
187 **Statistical analysis**
188
189 To evaluate possible differences in the percentage of each detected sub-haplotype (KArg-N1
190 and KArg-N2) among populations, Generalized Linear Mixed Models (GLMM) were
191 performed including the locality (*V. destructor* population) as a fixed factor and the colony
192 where mites were sampled as a random factor. The same analysis was performed for the
193 variable percentage of heteroplasmy. Normal distributions of means and residues were tested

194 using the Shapiro-Wilks and Levene tests. To obtain the most appropriate structure of variance,
195 the Akaike information criterion was used. To evaluate differences in both haplotypic and
196 heteroplasmy frequencies among localities, multiple comparisons were performed using Fisher
197 LSD ($\alpha = 0.05$).

198 Differences in haplotypic and heteroplasmy frequencies between regions (subtropical vs
199 temperate) were evaluated using X^2 test. To explore possible associations between the
200 haplotypic frequencies or heteroplasmy frequency of *V. destructor* with honey bee genetics (%
201 of African (A) lineage) and climatic and geographical variables (maximum and minimum
202 temperatures in January; longitude and latitude), Spearman correlations were calculated. The
203 climate information was obtained from the agro-meteorological stations closest to the sampled
204 localities using the SIGA system version 1.0.5 (SIGA, 2018) developed by INTA. Possible
205 differences in the percentage of colonies showing mites with different haplotypes (% of colonies
206 with co-infestation) among populations were evaluated using X^2 test. Statistical analyses were
207 performed with Statistica V 6.0 (Statsoft 2001) and InfoStat 2014 (Di Rienzo et al. 2014).

208

209 **Results**

210

211 **Sequence analysis and haplotype identification**

212

213 The sequence analysis of *cox1* (1166 nucleotides) confirmed the presence of the K haplotype for
214 all samples and evidenced 100% identity among them and with the sequences reported as K1-1
215 (GQ379056) and K1-2 (GQ379057) by Navajas et al. (2010) (Fig. S1).

216 A total of 2171 nucleotides were obtained from the amplicon sequencing corresponding
217 to *nad4, nad4-nad4L,* and *nad5* (Table S1). The comparison between the Argentinean and the
218 reference sequences revealed two and five punctual changes for *nad* regions compared to the
219 complete mitochondrial genome of *V. destructor* (AY163547.1, Evans and Lopez 2002, and
220 AJ493124.2, Navajas et al. 2002, respectively) (Table 2). These changes are synonymous
221 mutations.

222 Sequence analysis of *nad4* and *nad5* PCR fragments revealed 100% identity among the
223 Argentinean samples, without the presence of polymorphisms in the 1332 nucleotides analyzed
224 (Fig. S2, S3). Conversely, the sequence analysis of *nad4-nad4L* PCR fragments evidenced a
225 polymorphic site at 8694 nucleotide position (coordinate from AJ493124.1; Table 2). To
226 evaluate the distribution of this polymorphism, we increased the number of DNA samples. After
227 the analysis of 182 individuals, we detected that 73.6% of the samples (134 mites) were
228 identical to both reference sequences (AY163547.1 and AJ493124.2), hereafter named KArg-
229 N1 (Table 2). The remaining samples (26.4%, 48 mites) showed a different nucleotide at the
230 mentioned position or were heteroplasmic (see below, Table 2 and 3). The detected change
231 involves the change of G to A and represents a new variant of the K haplotype, hereafter named
232 KArg-N2 (Table 2, Fig. S4). While KArg-N1 sub-haplotype is present in all the analyzed mite
233 populations, KArg-N2 sub-haplotype is present in seven out of ten populations and at lower
234 frequencies (Fig. 1, Table 3). Although the percentage of the KArg-N2 sub-haplotype appears to
235 differ among localities, particularly between subtropical and temperate populations, these
236 differences were not statistically significant in the GLMM analysis (Table 3). However, a
237 significant association between the percentage of KArg-N2 sub-haplotype and population
238 latitude was found (Spearman correlation, coef: 0.63, P=0.05). The percentage of KArg-N1 sub-
239 haplotype differs among mite populations ($F_{9,30}$ = 154.27, P<0.0001, GLMM results). In
240 particular, Castelli and Leales populations showed the lowest percentages of KArg-N1, in
241 comparison with Formosa, which exhibited the highest frequency of this sub-haplotype
242 (multiple comparisons Fisher LSD; Fig. 1, Table 3). The other populations showed intermediate
243 values (Fig. 1, Table 3). In agreement with the results of KArg-N2, a border association
244 between the percentage of KArg-N1 sub-haplotype and population latitude was found
245 (Spearman correlation, coef: -0.58, P=0.08). No associations between the frequency of the mite
246 sub-haplotypes (KArg-N1 and KArg-N2) and the mitochondrial honey bee lineage of the colony
247 or other parameters (altitude, temperature) were found (data not shown).
248 Regarding the variability within colonies, the results indicated that mites with different
249 haplotypes coexist in the same colony for all the analyzed populations. The frequency of

250 colonies showing co-infestation varied between 33 and 75% (except for Formosa, where only
251 14% of the colonies had mites with different mt haplotypes), but showed no statistical
252 differences among populations (Chi-Square$_{(2)}$ = 5.7, P=0.22).
253
254 **Heteroplasmy analysis**
255
256 The chromatogram analysis of the obtained nucleotide sequences allowed us to identify double
257 peaks of similar height at position 8694 (corresponding to *nad4* gene, specifically to the sub-
258 haplotype defining site). The sequence analysis of cloned *nad4-nad4L* PCR fragments of the
259 samples showing double peaks confirmed the presence of site heteroplasmy. In all sampled
260 populations, 18.7% of mites containing two different *nad4-nad4L* sequences were detected
261 (those corresponding to KArg-N1 and KArg-N2), with percentages of heteroplasmy varying
262 among localities ($F_{9,30}$ = 9.29, P=0.0048, GLMM results, Fig. 1 and Table 3). Castelli and
263 Leales *V. destructor* populations showed the highest percentages of heteroplasmic individuals
264 (43.7% and 27.3%, respectively; Fig. 1 and Table 3) and were statistically different from the
265 other populations (P<0.001, Fisher LSD, GLMM results). In general, for temperate localities,
266 the frequency of heteroplasmic mites (7.7%) was lower than that for subtropical populations
267 (23.1%) (Chi-Square $_{(2)}$ = 5.79, P <0.05). Note that Formosa population exhibited a different
268 pattern from the other subtropical populations and did not present heteroplasmic individuals
269 (Fig. 1 and Table 3).
270 Additionally, other suggestive punctual changes, such as the presence of one
271 homoplasmic variant and the heteroplasmic configuration, were evident (Table S2). We
272 consider these positions “potentially heteroplasmic sites” due to the absence of the other
273 homoplasmic variant and the lack of confirmation by cloning. These changes were visualized in
274 the chromatograms and corresponded to 8390, 8529-8530, and 8645 coordinates from the
275 reference sequence of the *V. destructor* mitochondrial genome (AJ493124.1) (Table S2). These
276 potentially heteroplasmic sites (double peaks) are located within the *nad4* gene. Specifically, the
277 double peak at the 8390 position (C/A) was present in 12.2% of the samples in five out of ten

278 analyzed populations (Table 2, Table S2). We observed the presence of the remaining 87.8% of
279 individuals possessing C nucleotide, while individuals with A nucleotide at this coordinate were
280 not detected in the 182 analyzed sequences. This change is a non-synonymous mutation
281 producing a modification in the encoded amino acid glutamic acid (187:A) and generating a
282 stop codon (Fig. S5). The double peaks identified at 8529 and 8530 positions (AT/TA; Table 2)
283 were simultaneously detected in 8.8% of the samples and were evidenced for only two out of
284 ten mite populations (Table S2). This is a non-synonymous mutation that leads to the
285 substitution of asparagine (140:N) (an uncharged polar amino acid) for methionine (M) (a non-
286 polar aliphatic amino acid; Fig. S5). Finally, the double peak at 8645 nucleotide positions (A/T)
287 was present only in one of the samples, belonging to a colony from Reconquista (Santa Fe
288 province) (Table 2; Table S2). This is a non-synonymous mutation that leads to the substitution
289 of phenylalanine (102:F) (an amino acid from the group of aromatics) for isoleucine (I) (a non-
290 polar aliphatic amino acid; Fig. S5).
291
292 **Discussion**
293
294 This study provides valuable genetic information about *V. destructor* populations present in
295 managed honey bee colonies from the most important apicultural regions of Argentina. By
296 using PCR and sequencing of four regions of the mtDNA, we analyzed the genetic diversity in
297 ten mite populations from *A. mellifera* apiaries located in both temperate and subtropical
298 regions of the territory, previously unexplored. Our data are in line with the low genetic
299 variability described for this species (e.g. Solignac et al. 2005: Navajas et al. 2010) but reveal
300 novel genetic variation for the new mtDNA marker studied here (ND4 gene) and disclose the
301 presence of site heteroplasmy, which varies in frequency across mite populations.
302 The genetic variability of *V. destructor* worldwide populations has been extensively
303 studied using mitochondrial haplotypes based on the analysis of a region (458 bp) of
304 *cytochrome oxidase 1* gene (Solignac et al. 2005; Warrit et al. 2006; Muñoz et al. 2008;
305 Strapazzon et al. 2009; Navajas et al. 2010). Based on this marker, two mitochondrial

306 haplotypes (K1 and J1) have been reported infesting *A. mellifera* colonies (Navajas et al. 2010).
307 Here, based on the sequence analysis of *cox1* (1116 bp), the exclusive presence of the K1 and
308 100% of sequence identity were detected among the analyzed populations of *V. destructor* from
309 Argentina. Consistently with Maggi et al. (2012), we found no presence of J haplotype in these
310 Argentinean populations of the mite, reinforcing the evidence of the limited occurrence of this
311 haplotype on restricted areas of Brazil (Fernando de Noronha Island) (Guerra et al. 2010). The
312 negligible variability identified for *cox1* region among our populations is consistent with
313 previous results in other populations of the mite infecting *A. mellifera* (Solignac et al. 2005;
314 Muñoz et al. 2008; Navajas et al. 2010; Gajic et al. 2013, 2016; 2019; Salvaé et al. 2016;
315 Kelomey et al. 2017) and supports the hypothesis of a bottleneck that occurred at the beginning
316 of the invasion to the western honey bee as previously suggested by Solignac et al. (2005).
317 Moreover, additional genetic bottlenecks could have occurred as *V. destructor* spread around the
318 world (see Roberts et al. 2015).
319 Although the low genetic variability seems to be a general trend in *V. destructor*, the
320 analysis of new mitochondrial markers can be useful for detecting novel genetic variation (e.g.
321 Navajas et al. 2010; Gagic et al. 2013) and monitoring relevant traits in mite populations, such
322 as acaricide resistance (see Beaurepaire et al. 2017). Our results, based on the sequence analysis
323 of *nad4* gene, allowed us to identify two K1 sub-haplotypes (KArg-N1 and KArg-N2) in our
324 populations. KArg-N2 corresponds to a new variant reported for the K1 haplogroup, and it is
325 present in most of the Argentinian populations of the mite analyzed here. Compared to the
326 previously described sequences from mt genome of mites with K haplotype (Evans and Lopez
327 2001; Navajas et al. 2002) and to KArg-N1, the KArg-N2 sub-haplotype exhibits a single-point
328 synonymous mutation in the *nad4* sequence at the 8694 nucleotide position (AJ493124.1). This
329 new variant appears to be well-established in honey bee colonies, since it is widely distributed
330 across populations and present in relatively high frequencies. This can be explained by the
331 inbred and haplodiploid reproductive system of the mite that tends to fix novel mutations
332 (Solignac et al. 2005; Rosenkranz et al. 2010; Nazzi & Le Conte 2016) and by the posterior

333 within-colony mite genetic exchange and between-colony mite transmission driven by natural
334 bee movement or human-induced activities (Dynes et al. 2017, discussed below).

335 Transhumance is a habitual human activity in which colonies are moved for crop
336 pollination purposes or in search of earlier flowering areas for colony multiplication. Every
337 year, beekeepers from temperate regions (mainly from Buenos Aires province) move colonies
338 from the south (temperate climate) to the north (subtropical climate) of the territory to find
339 better conditions for colony multiplication and gain advantages at initial stages of the honey
340 production season (Agra et al. 2018). This practice, together with the commerce of nuclei and
341 package bees between different regions, contributes to the gene flow among honey bee
342 populations and consequently leads to interactions between mites of different colonies
343 parasitizing them. We found that the frequency of the new sub-haplotype differs among the
344 analyzed populations and showed a positive association with latitude. In this scenario, we
345 localize the putative origin of KArg-N2 sub-haplotype in northern Argentina. It either originated
346 in this area or was introduced from a neighboring country and then spread to the southern
347 populations, possibly mediated by honey bee colony movements. However, the present results
348 do not indicate whether the detected sub-haplotype originated in Argentinian populations or is
349 "ancestral" to the American populations of the mite. The analysis of this marker in other
350 populations of Brazil, Uruguay, or the region will generate information about the origin and
351 subsequent dispersion of this new variant.

352 In addition to human influence through transhumance and commercial activities, the
353 honey bee genetic lineage could contribute to explaining the pattern of sub-haplotype
354 frequencies. Although we did not find a significant association between the honey bee
355 mitochondrial lineage and the mite sub-haplotype frequency, we did find an association between
356 the mite sub-haplotype and latitude of the mite population. In a related work on genetic
357 variability of honey bees from Argentina, Agra et al. (2018) detected a latitudinal cline from
358 north to south for the level of hybridization between Africanized and European honeybees based
359 on the use of highly polymorphic markers (microsatellites). Further genetic analysis of honey
360 bee colonies from our study using microsatellites or other kind of highly polymorphic markers

361 could potentially show any association between mite haplotype and honey bee lineage
362 considering the latitudinal cline and other factors involved in seasonal cycle and mite
363 dispersion.

364 We found within-colony genetic diversity for all the analyzed populations. In fact, this
365 situation, in which mites with different haplotypes coexist in the same colony, appears to be
366 common in our *V. destructor* populations. These results are in line with Dynes et al. (2017),
367 who found a population structure for the mite with genetically distinct individuals coexisting in
368 the same colony and probably more sexual outcrossing than previously expected (see
369 Dietemann et al. 2019). As proposed by these authors, one potential explanation of this diversity
370 is the within-colony genetic exchange between mites (Dynes et al. 2017). Although most
371 matings in *V. destructor* occur between siblings, outcrossing can occur when more than one
372 foundress mite enters the same brood cell to lay eggs. This scenario occurs more frequently at
373 specific points in the annual cycle when the ratio of mites to bee brood is high, as in late
374 summer (Dynes et al. 2017; Beaurepaire et al. 2017). In fact, in temperate climates of Argentina,
375 multiple mite infestations are frequently detected in worker brood cells during early autumn and
376 in drone brood cells during the spring (Muntaabski, personal communication), possibly
377 contributing to within-cell outcrossing.

378 Heteroplasmic mites were unambiguously identified in several of the analyzed samples
379 from the Argentinian populations of *V. destructor*. These findings are in line with preliminary
380 results by Navajas et al. (2002), who suggested the existence of heteroplasmy in *V. destructor*
381 and with a more recent study performed in Serbian populations of the mite (Gagic et al. 2016).
382 Using ARMS and RFLP methods, these authors detected site heteroplasmy within *cox1* and *cytb*
383 sequences at haplotype defining sites, particularly for the S1 and P1 haplotypes described for
384 the region (Gajic et al. 2013; 2016; 2019). In this sense, the present study reports the first
385 occurrence of site heteroplasmy within *nad4* sequence in this species (and for K1 haplotype)
386 and suggests that this phenomenon would be relatively frequent in mites. Although the
387 penetrance of heteroplasmy varied among our populations, the results seem to support the

388 stability of this phenomenon in the analyzed mite populations, but this finding must be further
389 evaluated.
390 Heteroplasmy has been explained by different mechanisms in different species (Wolf et
391 al. 2013; Xiong et al. 2013; Robinson et al. 2015) and is linked to the inherently high mutation
392 rate of mtDNA, the error-prone nature of DNA polymerase, and the lack of DNA repair
393 mechanisms within the mitochondria (Avise 2000; Chinnery et al. 2000; Wolf et al. 2013).
394 Mutations may arise during gametogenesis and embryonic development, generating unique
395 haplotypes. If mutations are generated within female germ cells, these new mitochondrial
396 haplotypes can be transmitted to the offspring if they persist through the germ-line bottleneck
397 associated with oocyte formation and escape selection against deleterious mutations (Chinnery
398 et al. 2000; White et al. 2008). The mutation phenomenon in the mitochondria with a
399 subsequent passage through the generations appears to be a plausible mechanism that generates
400 heteroplasmy in *V. destructor*, since it is common in other arthropod ectoparasites of the
401 Subclass Acari (Van Leeuwen et al. 2008; 2010).
402
403 **Conclusion**
404
405 In the present study, we identified a new polymorphic mtDNA marker (ND4) to analyze the
406 genetic variability in *V. destructor* populations. The presence of site heteroplasmy and the
407 identification of a new sub-haplotype in Argentinean *V. destructor* populations using this new
408 molecular tool provide useful information for further analysis. The study of behavioral and
409 population parameters of the identified mite sub-haplotypes, such as reproduction and virulence
410 to local honey bee colonies, will represent a future challenge and will contribute to
411 understanding the dynamic interaction between the mite and honey bee populations. In addition,
412 for exhaustive monitoring of sub-haplotypes distribution, its temporal stability in honey bee
413 colonies and its potential influence on apiculture will require further attention. Our findings and
414 perspectives would be useful to apply in other *V. destructor* populations and to implement more
415 specific and efficient control strategies against varroosis.

416

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423

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428

429 **Author contributions**

430 ACS, SBL, MAP and JLC conceived the study; IM conducted the experiments and analyzed the
431 data; MCL provided data analysis support; RMR provided experimental support; IM, ACS, and
432 SBL wrote the manuscript, and all authors accepted the final version of the manuscript.

433

434 **Compliance with ethical standards**

435 On behalf of all authors, the corresponding author states that there is no conflict of interest.

436

437 **References**

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