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Genetic diversity of *Varroa destructor* parasitizing *Apis mellifera unicolor* in Madagascar

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Abstract – *Varroa destructor* is an invasive alien species that has been reported parasitizing the endemic honey bee of Madagascar, *Apis mellifera unicolor*, since 2010. Studying its nuclear genetic diversity and structure was our main goal. Using 11 microsatellite loci and 344 mites collected from 12 apiaries, we observed a low genetic diversity, with only 8 multilocus genotypes (MLG) identified. *V. destructor* populations form a single genetic cluster, clonal richness ranged from 0.02 to 0.20, and number of MLG within apiaries varied between one and six MLGs. About 69.5% of the mites analyzed harbored the same genotype (100%, homozygous), and 23.3% had a genotype differing by a single allele. The overall low diversity observed suggests one or multiple introductions of similar genotypes. The greater abundance of MLGs in High Land apiaries (eight MLGs) than on the east coast (two MLGs) and the presence of particular MLGs in High Land apiaries favor the hypothesis that *V. destructor* has been first introduced close to the international airport, and then spread to other regions by commercial exchanges.

microsatellite / multilocus genotypes / *Varroa destructor* / Madagascar

1. INTRODUCTION

Invasive species are known as a leading threat to biodiversity as well as an increasing economic concern (Wilcove et al. 1998; Pimentel et al. 2000). Especially when invasive species are parasitizing endemic generalist pollinators in hotspot of biodiversity implying their decline, it could have dramatic ecologic and economic impact (Potts et al. 2010).

Studies about genetic diversity of invasive populations can provide an indication of the amount of genetic variation lost during the colonization bottleneck and can provide evidence of multiple sources of introduction (Sakai et al. 2001). At the founding stage, the number of individuals introduced is usually small and contains only a low portion of the “genetic pool” present in the area of origin (Lee 2002). Furthermore, the level of genetic diversity in the invaded zone depends on the contribution of individuals from different populations of the source area. Indeed, when the introduced individuals originated from genetically heterogeneous populations, genetic mixing between populations in the new environment colonized could generate a higher genetic diversity (Dlugosch and Parker 2008). The reproduction system of the introduced species plays also an important role in modulating the genetic diversity (Lynch 1984).

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Although *Varroa destructor* (Mesostigmata: Varroidea) is an obligate ectoparasite mite and shows a sexual reproduction mode, mating takes place between brother and sisters, increasing inbreeding. This reproduction system (adelphogamy), coupled with the haploid-diploid (De Ruijter and Pappas 1983; Martin et al. 1997) and pseudo-arrhenotoky sex determination systems (Martin et al. 1997), are responsible for the observed high deficit of heterozygotes and greatly increases the fixation rate of mutations (Cornuet et al. 2006). In case of multi-infested honeybee brood cells by *V. destructor*, particularly when heavy infestation of the colony is observed, male could mate with non-sisters (female offspring from other female founder) and it could limit the inbreeding, permitting genetic mixing within populations (Solignac et al. 2005).

Spread of honeybee subspecies has facilitated the transfer of *V. destructor* from one host to the other. *V. destructor* shifted from its original Asiatic host *Apis cerana* (Hymenoptera: Apoidea) and invaded the subspecies *Apis mellifera* (Denmark et al. 1991; Frazier et al. 2010). The first observation of *V. destructor* on *A. mellifera* was reported in 1957 in Asia, then, this invasive alien species (Handley et al. 2011; Barlow et al. 2002) became one of the most common pests of *A. mellifera*.

V. destructor was first reported in Madagascar in 2010 (OIE- Feb-2010; Rasolofoarivao et al. 2013) and has been observed parasitizing *Apis mellifera unicolor* (Latreille 1804) the endemic honey bee (Ruttner 1988), of the African lineage (Franck et al. 2001; Rasolofarivao et al. 2015b). The impact of the parasite on *A. m. unicolor* was severe (Rasolofoarivao et al. 2013, 2015a), despite its African genetic background. Indeed, it was reported that populations of A lineages, and notably *Apis mellifera scutellata*, were able to better tolerate *V. destructor* infestation (Strauss et al. 2015, 2016; Mortensen et al. 2016). Nevertheless, the spread of the mite is still relatively limited to the High Lands and the east coast regions (Rasolofarivao et al. 2015b). In this work, we examined the nuclear genetic diversity of *V. destructor* in Madagascar with microsatellite markers.

2. MATERIALS AND METHODS

2.1. *V. destructor* sampling

Sampling of phoretic *V. destructor* was carried out from January 2011 to January 2012 in 69 managed colonies from 12 infested apiaries (Figure 1, Table I) of the endemic honeybee subspecies *A. M. unicolor* (Rasolofoarivao et al. 2015b). Two apiaries were located on the east coast and 10 in the High Lands region. Worker honeybees were randomly sampled inside the hive and preserved in 96% ethanol. Number of workers sampled per apiary varied between 2 and 315. In the laboratory, tubes were vigorously shaken to dislodge *V. destructor* from the bodies of the worker, and each worker was visually inspected to remove any mites on the bee's surfaces. Samples were stored separately in a new tube with 96% ethanol and frozen at -20°C until they were processed.

2.2. DNA extraction and genotyping of *V. destructor*

Total DNA of 480 *V. destructor* was isolated using the protocol of extraction as previously described (Delatte et al. 2010); it consists in extracting DNA from whole mite by crushing them individually in a sodium dodecyl sulfate (SDS)/Proteinase K buffer and precipitating DNA in ethanol, then elute it in pure water.

DNA of each sample was amplified at 12 microsatellite loci: two (vj292, vj295) among those published by (Evans (2000) and 10 (VD001, VD016, VD112, VD114, VD119, VD126, VD146, VD152, VD154, VD163) by Solignac et al. (2003). PCR reactions were performed in a 10- μL final reaction volume using two primer mix and Type-it Multiplex PCR Master Mix (QIAGEN) kits. PCR program was run with an initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, annealing for 1 min at 55°C , and then elongation at 72°C for 1 min. A final extension step was done at 72°C for 10 min. PCR products were then prepared for capillary electrophoresis and genotyped using an automated DNA sequencer by Applied Biosystems (AB). Electrophoresis profiles were obtained by AB

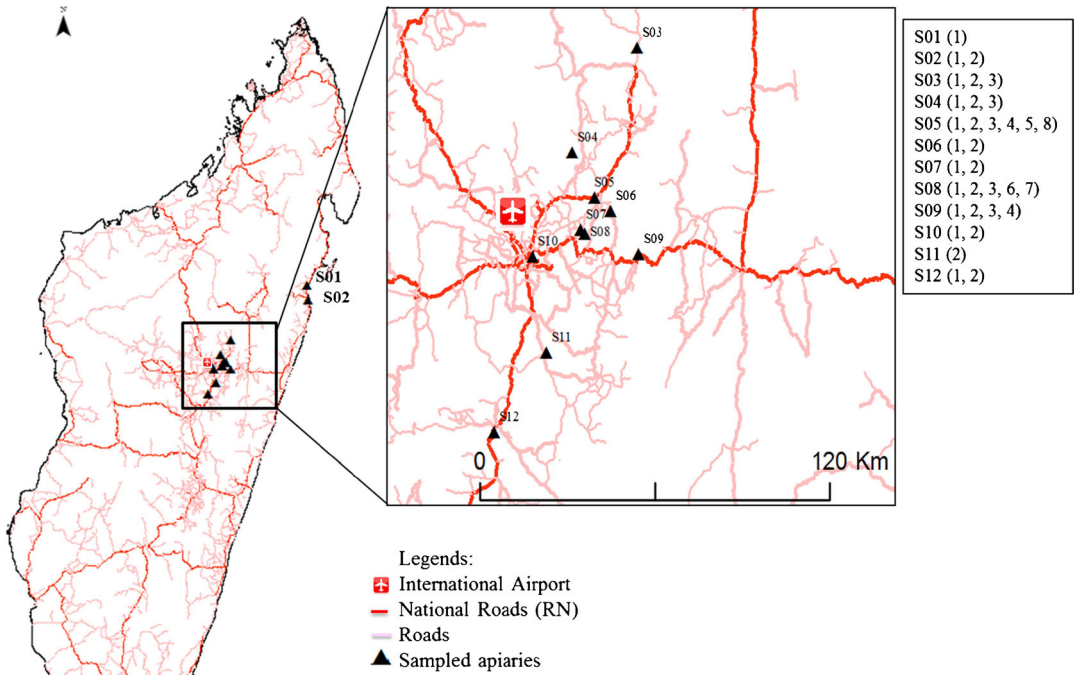


Figure 1. Location of the 12 apiaries sampled to study genetic diversity and structure of *V. destructor*. For each apiary (names indicated in Table I), the different multilocus genotypes (MLGs) observed are given (at right).

3130XL data collection. Allele identification at each locus was performed by comparison with

the size standard using the Genemapper V. 4.0 software (AB).

Table I. Apiary information for each sampled apiary is given: number of colonies and mites collected (number in parenthesis refers to individuals that have not been used for molecular analysis), sampling years and the multilocus genotypes (MLG)

Apiaries codes	Apiaries' location	Number of colonies	Number of mites	Sampling year	MLG detected
S01	Rantolava	1	1	2012	1
S02	Mahambo	2	5	2012	1, 2
S03	Masakalina	4	7	2012	1, 2, 3
S04	Ambohitrolomahitsy	6	15	2012	1, 2, 3
S05	Amby	(3) 11	(103) 136	2011	1, 2, 3, 4, 5, 8
S06	Ambotseheno	6	8	2012	1, 2
S07	Anjepy	7	13	2012	1, 2
S08	Betoho	(4) 11	(110) 231	2011	1, 2, 3, 6, 7
S09	Mandraka	1	20	2012	1, 2, 3, 4
S10	Ankatso	(9) 2	(12) 9	2011	1, 2
S11	Andramasina	1	6	2012	2
S12	Ambatolampy	1	29	2012	1, 2

2.3. Population genetic analysis

Individuals with missing genotype data were excluded from analysis. Only apiaries with at least 10 samples were considered for the population genetics analysis. All pairs of loci were tested for linkage disequilibrium using the probability test in Genepop V. 4.2 (Rousset 2008). Gene diversity within populations was estimated using observed (H_O) and expected (H_E) heterozygosity implemented in Genepop V.4.2 (Rousset 2008). Single and multilocus Fixation Index (F_{IS}) were estimated through the fixation index (Weir and Cockerham 1984). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested using a two-tailed Fisher's exact test based on Markov-chain randomization (1000 dememorizations with 100 batches and 1000 iterations per batch (Rousset 2008). As the number of alleles observed within a given population could be positively affected by the sample size (Leberg 2002), allelic richness, adjusted by the lowest sample size (Leberg 2002), was calculated using Fstat V. 2.9 (Goudet 2001). Weir's pairwise F_{ST} (Weir and Cockerham 1984) were calculated across all population pairs in order to assess genetic differentiation between apiaries.

Levels of population admixture were quantified through a number of Bayesian clustering procedures implemented in Structure 2.3.4 (Pritchard et al. 2000). Structure calculates clustering patterns solely based on MLG (Falush et al. 2007). The number of clusters was inferred according to Evanno et al. (2005), and the ad hoc statistic was calculated for K ranging from 1 to 20. Structure was run for 1 million generations (burn-in = 100,000 generations) with 10 iterations for each value of K . Ad-Hoc statistic was processed through the Structure harvester website (<http://taylor0.ucla.edu/structureHarvester/>).

2.4. MLG diversity

Multilocus genotypes (MLGs) were assigned using the GenClone V. 2.0 software (Arnaud-Haond and Belkhir 2007). Spearman's rank test was used to test correlation between sample sizes and number MLG found per site. Level of clonality was estimated using genotypic richness

(R), with $R = 0$ when population consists in a single MLG, and 1 when each individual possesses a unique MLG. The Simpson index (D , also known as Nei's genetic diversity), with values corrected for finite sample sizes, was calculated (D ranging from zero to one, with higher values corresponding to greater clonality diversity). Finally, the Evenness (E) which is the measure of how MLG are distributed and their abundance among sample was also calculated (E ranging from 0 to 1, with 1 indicating that all MLGs have equal abundance). All indices were calculated per apiary using the GenClone V. 2.0 software (Arnaud-Haond and Belkhir 2007).

Matrix of pairwise distances between distinct MLG was used to construct a minimum spanning network using the NETWORK 4.6.1.2 software (Polzin and Daneshmand 2003).

3. RESULTS

3.1. Genetic diversity

Of the 480 mites analyzed, 344 were successfully genotyped at 11 of the microsatellite loci. Among the set of 11 primers considered, only 3 were polymorphic (VD001, VD112, vj295). Number of alleles at the three polymorphic loci was two alleles for VD112, three for VD001, and four for vj295 (Table II). A total of 11 alleles across the 11 loci were congruent with previous studies on *V. destructor* worldwide populations (Table II) by Evans (2000) and Solignac et al. (2003). New alleles such as 125 and 127 were found in locus VD001, allele 110 in VD112, and allele 140 and 143 in vj295. No significant linkage disequilibrium between pair of loci was detected (Fisher's exact test, $P > 0.05$).

3.2. Clonal diversity

A total of eight MLGs were found and coded from 1 to 8 (Table II). MLGs were not equally represented (Figure 2). MLGs 1 and 2 account for 92.8% of the data set, 69.5 and 23.3%, respectively (Table II). The most frequent MLG (1) was homozygous at all loci.

Six MLGs (2, 3, 5, 6, 7, and 8) were heterozygous at one locus only, deriving from one

Table II. Observed microsatellite multilocus genotypes (MLG) and allele size (pb) at each locus

MLG	VD001	VD016	VD114	VD146	VD152	vi292	vi295	VD112	VD119	VD154	VD163	Percent
1	155/155	270/270	221/221	174/174	224/224	233/233	155/155	140/140	120/120	127/127	112/112	69.48
2	155/155	270/270	221/221	174/174	224/224	233/233	143/155	140/140	120/120	127/127	112/112	23.26
3	155/155	270/270	221/221	174/174	224/224	233/233	155/155	110/140	120/120	127/127	112/112	4.94
4	155/155	270/270	221/221	174/174	224/224	233/233	143/143	140/140	120/120	127/127	112/112	1.16
5	155/155	270/270	221/221	174/174	224/224	233/233	140/155	140/140	120/120	127/127	112/112	0.29
6	155/155	270/270	221/221	174/174	224/224	233/233	147/155	140/140	120/120	127/127	112/112	0.29
7	125/125	270/270	221/221	174/174	224/224	233/233	155/155	110/140	120/120	127/127	112/112	0.29
8	127/155	270/270	221/221	174/174	224/224	233/233	155/155	140/140	120/120	127/127	112/112	0.29
Nb _a	3	1	1	1	1	1	4	2	1	1	1	

Bold font indicates MLG allelic variations. Frequency of each MLG detected in the whole dataset (344 samples) was presented in percentage (%). Nb_a, the number of allele per locus

mutation event from the dominant one (except MLG 7, with three alleles distinct from MLG 1, Figure 2). MLG 4 was also homozygous at all loci, showing two allele differences with MLG 1 (Figure 2). Distribution of the pairwise number of allele differences between MLGs in the whole data set appeared uni-modal with one to five alleles of difference between MLGs (Figure 2).

The number of MLGs per apiaries ranged from one (S01 and S11) to six (S05). The two most frequent MLGs (1 and 2) were found in all apiaries (except in S01 for MLG 1 and S11 for MLG 2). All MLGs were identified in the High Lands region. In contrast, on the east coast, with only six mites analyzed, only two MLGs were detected (MLG 1 and MLG 2). Four MLGs were detected only once, MLG 5 and MLG 8 in S05 and MLG 6 and MLG 7 in S08, both apiaries being located next to the international airport and were also the most extensively sampled sites (Table III).

3.3. Structure of the genetic diversity within and among apiaries

Mean allelic richness within apiaries was very low and close to 1 (1.09–1.18, see Table III). Levels of expected heterozygosity were close to

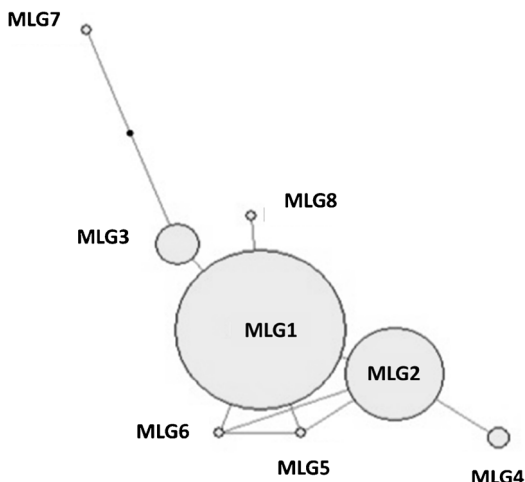


Figure 2. Minimum spanning network of *V. destructor* genetic distances computed as number of allele differences between MLGs (11 microsatellite loci). Each circle represents a different MLG, circle size is proportional to sample size, and line between the circles indicates a difference of one allele.

Table III. Descriptive statistics of genetic and clonal diversity across all loci (11 microsatellite loci) for 6 of the 12 populations (i.e., only apiaries with more than 10 individuals successfully genotyped were presented)

Apiary	Number	<i>G</i>	<i>R</i>	<i>D</i>	<i>E</i>	<i>Ar</i>	<i>H_E</i>	<i>H_O</i>	<i>F_{IS}</i>
S04	15	3	0.14	0.53	0.60	1.18	0.03	0.03	-0.06
S05	64	6	0.08	0.26	0.16	1.13	0.01	0.01	0.18
S07	12	2	0.09	0.48	0.84	1.09	0.04	0.06	-0.47
S08	183	5	0.02	0.43	0.51	1.14	0.02	0.02	-0.07
S09	16	4	0.20	0.71	0.80	1.18	0.05	0.03	0.42
S12	22	2	0.05	0.41	0.75	1.09	0.02	0.02	-0.13
	312	8							
Overall	344	8	0.02	0.46	0.50	1.16	0.03	0.03	-0.02

Number number of samples, *G* number of MLGs detected

R clonal richness index as $R = (G - 1)/(N - 1)$ (Dorken and Eckert 2001)

D Simpson's index (Arnaud-Haond et al. 2007)

E evenness (Arnaud-Haond et al. 2007)

Ar allele richness adjusted for sample size

H_E unbiased expected heterozygosity (Nei 1987)

H_o observed heterozygosity

F_{IS} inbreeding coefficient, all

F_{IS} are not significant departures from HWE ($P \geq 0.05$)

zero, ranging from 0.01 to 0.05 (Table III). The *F_{IS}* ranged from -0.47 to 0.42; the Hardy-Weinberg Equilibrium were not found in all tested populations ($P \geq 0.05$).

Overall clonal richness was of 0.02 and ranged from 0.02 to 0.20 within apiaries. Overall Simpson's index was 0.46. The lowest Simpson's index was found in S05 ($D = 0.26$) and the highest in S09 ($D = 0.71$). Overall Evenness index was 0.50 and ranged from 0.16 to 0.84 within populations, i.e., MLGs were unequally distributed (Figures 1 and 2). MLGs richness within apiaries (Table III) was not consistently correlated with sample size (*N*) (Spearman test, $P = 0.76$), for instance, apiary S09 ($N = 16$) had four distinct MLGs and consequently the highest clonal richness ($R = 0.20$) and allelic richness adjusted for sample size ($Ar = 1.18$).

The genetic differentiation among apiaries, estimated as *F_{ST}*, ranged from -0.03 to 0.29, with pairwise *F_{ST}* estimates being significant for four pairs of populations (S07 with S04 and S05 with S07, S08, S09, $P < 0.05$; Table IV).

Based on the individual Bayesian clustering method implemented in the STRUCTURE software (Supplementary Data, Figure S1), no further recognizable population groups were observed, all the samples both from the High Lands and east coast were considered as a single genetic cluster.

Table IV. Genetic differentiation (*F_{ST}*) between each pair of populations estimated across all loci, (i.e., apiaries with more than 10 individuals successfully genotyped)

Apiary	S04	S05	S07	S08	S09
S05	0.01				
S07	0.16*	0.29*			
S08	0.02	0.02*	0.13		
S09	0.06	0.19*	-0.03	0.07	
S12	0.03	0.04	0.09	-0.01	0.03

Significant pairwise *F_{ST}* comparisons at the $P < 0.05$ level are indicated with asterisk

4. DISCUSSION

4.1. Introduction of *V. destructor* in the island

After the first description of *V. destructor* in Madagascar in 2010, both on the east coast close to the main port of the island and in the area close to the airport (High Lands), the mite has spread to neighboring districts. These two simultaneous mite outbreaks observed 400 km apart could have either resulted from two independent infestations of *V. destructor* on the island, or from one single event followed by honey bee colony exchanges between the east coast and the capital areas (Rasolofoarivao et al. 2013). Cytochrome oxidase I (COI) gene analysis showed that all mites were of the Korean strain (K1-1) with no genetic variation (Rasolofoarivao et al. 2013). Our nuclear genetic results showed that the same homozygous genotype was overrepresented (almost 69.5% for MLG 1), and the second most common one (MLG 2) differed by 1 allele only, both were widespread in all apiaries and at the center of the MLG network. Moreover, we found no evidence of admixture from multiple founding populations in the Bayesian cluster analysis, suggesting that *V. destructor* invasion likely result from one major introduction event.

The greater abundance of MLGs in the High Lands (eight MLGs) than on the east coast (only two MLGs) and the presence of particular MLGs in the capital apiaries favor the hypothesis that *V. destructor* has been first introduced in apiary (ies) close to the international airport, and then spread to other regions by commercial exchanges, as most of the goods transit in the capital.

Gene diversity was very low, and the observed heterozygosity was of 3% within population. Solignac et al. (2005) also found a low heterozygosity of less than 1.3% (using 13 microsatellites) in 45 populations of mites ($n = 565$) from 17 countries (newly infested countries). Similar results were found by Strapazzon et al. (2009) using four loci in *V. destructor* parasitizing Africanized honeybee subspecies. The low genetic diversity in *V. destructor* populations affecting *A. M. unicolor* could be the effect of founding events along the migration to Madagascar and potentially

accentuated by anthropomorphic activities (such as mite control, beekeeping practices) in apiaries. As suggested by Solignac et al. (2003), reduced genetic diversity detected in *V. destructor* populations may result from a severe bottleneck, which probably occurred at the time of parasite transfer from *A. cerana* to *A. mellifera* (Solignac et al. 2003, 2005). Moreover, the reduced nuclear genetic diversity of *V. destructor* in newly invaded subspecies of *A. mellifera* could be explained by a second strong founder effect (Solignac et al. 2003).

Furthermore, the low level of heterozygosity detected may be explained by autozygosity, which results from recurrent sib-mating (Solignac et al. 2005). The majority of Malagasy beekeepers do not practice transhumance; this factor could limit both ectoparasite spread and multi-infestation of honeybee brood cells by genetically distinct females. A recent study by Dynes et al. (2017) using 10 microsatellite markers on mite populations sampled from apiaries where the first mite infestation occurred more than 30 years ago (in Georgia, USA), showed higher genetic diversity than in other studies made on this parasite. Those results suggest that mite transmission between colonies is maintained by multiple sustained events rather than rare single transmission events. This phenomenon will participate in increasing population diversity, more than previously expected in new invaded areas, implying higher diversity in the future is to be expected in Madagascar, such as its rapid spread and potential apparition of acaricide.

Interestingly, only two MLGs were detected in the population with the lowest *V. destructor* infestation rate, against five and six MLGs detected in the two other apiaries, S08 and S05, respectively. This could be interpreted as genetic diversity arising from high mite fertility and numerous reproductive events. Indeed as underlined by Solignac et al. (2005), heterozygosity in that particular case could be generated by crosses between two lineages in the case of two foundresses entering the same cell.

In conclusion, low levels of genetic diversity were found in *V. destructor* parasitizing *A. m. unicolor*, which suggest one or multiple introductions of similar genotypes through the airport.

These data are crucial for the governmental agency to emphasis controls (i) within the country between districts to lower the spread of the mite at the maximum and (ii) at the custom to prevent more introductions from abroad.

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AUTHORS' CONTRIBUTIONS

HD and JC designed protocols; HR did the fieldwork; AS and HR did the lab work; HR, HD, and JC did the analyses; and HR, HD, JC, BR, and LHRR interacted for writing the manuscript.

Diversité génétique de *Varroa destructor* parasitant *Apis mellifera unicolor* à Madagascar

Microsatellite / génotype multi-loci / Acari / Apidae / Madagascar

Genetische Diversität von Varroamilben (*Varroa destructor*), die *Apis mellifera unicolor* in Madagaskar parasitieren

Mikrosatelliten / Multi-Locus-Genotypen / Acari / Madagaskar

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