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The invasive Korea and Japan types of *Varroa destructor*, ectoparasitic mites of the Western honeybee (*Apis mellifera*), are two partly isolated clones

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Varroa destructor, now a major pest of the Western honeybee, *Apis mellifera*, switched from its original host, the Eastern honeybee, *A. cerana*, *ca*. 50 years ago. So far, only two out of several known mitochondrial haplotypes of *V. destructor* have been found to be capable of reproducing on *A. mellifera* (Korea and Japan). These haplotypes are associated in almost complete cytonuclear disequilibrium to diagnostic alleles at 11 microsatellite loci. By contrast, microsatellite polymorphism within each type is virtually absent, because of a severe bottleneck at the time of host change. Accordingly, 12 mitochondrial sequences of 5185 nucleotides displayed 0.40% of nucleotide divergence between haplotypes and no intra haplotype variation. Hence, each type has a quasi-clonal structure. The nascent intratype variability is subsequent to the clone formation 50 years ago: in both types the variant alleles differ from the most common by one (in 10 cases), two (five cases) or three (one case) repeated motifs. In addition to individuals of the two 'pure' types, five F_1 hybrids and 19 recombinant individuals (Japan alleles introgressed into the Korea genetic background) were detected. The existence of F_1 and recombinant individuals in admixed populations requires that double infestations of honeybee cells occur in a high proportion but the persistence of pure types suggests a post-zygotic isolation between the two clones.

Keywords: *Varroa destructor*; microsatellites; mtDNA; clonal structure; sexual isolation; invasive species

1. INTRODUCTION

During the past 30 years, varroatosis has become the most damaging pest for beekeeping. The acari responsible for this disease was first described from Java by Oudemans (1904) as a parasite of the Eastern honeybee, *Apis cerana*, and was named *Varroa jacobsoni*. This natural parasite of *A. cerana* shifted to the occidental honeybee, *A. mellifera*, when the latter was introduced into Asia for apicultural reasons. On the two host species, the parasite was first considered as belonging to the species *V. jacobsoni*. However, a detailed study based on mitochondrial DNA sequences (Anderson & Trueman 2000) revealed the existence of at least two species: *Varroa jacobsoni sensu stricto* is present in Indonesia and Malaysia on *A. cerana* whereas the newly described species, *V. destructor*, is mainly found on *A. cerana* from continental Asia. Only *V. destructor* parasitizes *A. mellifera*, despite all publications before 2000 (before the nomenclature change) referring to *V. jacobsoni*. In the Western honeybee, two *V. destructor* mitochondrial haplotypes were detected: the Korean (K) and the Japanese (J) haplotypes, named from the countries in Asia where they were first detected on their native hosts, *A. cerana*. Whereas the K haplotype is present on *A. mellifera* almost worldwide (including most regions of Asia), the J haplotype is present only in Japan, Thailand and the Americas.

The interspecific host transfer seems to have occurred at least on two occasions (Ruttner 1983; de Guzman *et al.* 1997; Oldroyd 1999). In Japan, *A. mellifera* was introduced in 1877 (Sakai & Okada 1973) but the occurrence of *Varroa* on the Western honeybee was not reported until 1957. From Japan, the parasite invaded Paraguay in 1971, Brazil in 1972 and later North America (de Jong *et al.* 1982). A second invasion pathway occurred when colonies of *A. mellifera* from Ukraine were introduced to the far east of the Soviet Union, in the neighbourhood of Vladivostok, allowing the shift of *Varroa* infecting local colonies of *A. cerana* onto *A. mellifera*. Infected colonies were later found in European USSR in 1975 (Crane 1978), and from

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there the parasite invaded the rest of Europe, followed by a rapid and almost worldwide expansion.

Morphological (Grobov *et al.* 1980; Delfinado-Baker & Houck 1989), allozymic (Issa 1989; Biasiolo 1992) and cuticular hydrocarbon (Nation *et al.* 1992) analyses have evidenced some differences mainly between the two types, but very few, if any, within each type.

Randomly amplified polymorphic DNA (RAPD) markers (Kraus & Hunt 1995) also displayed a very low genetic variation within populations. Subsequent RAPD surveys distinguished two types of *Varroa* (de Guzman *et al.* 1997): one, the R (for Russian) type corresponded to mites from the United States, Russia, Morocco, Germany, Italy, Spain and Portugal populations and the other, the J (for Japan) type, to populations from Japan, Brazil and Puerto Rico and an admixture of both in North America (de Guzman *et al.* 1999). These two types carry the Korea and Japan haplotypes, respectively, and following Anderson (2000), are hereafter referred to as Korea or K (= Russia) and Japan or J types. The K type is highly virulent on *A. mellifera*, but the J type is far less aggressive (Delfinado-Baker 1988).

The aim of this study was to gain insight into the invasion process of *V. destructor* at both worldwide and local scale and on the interaction between the K and J types. We took advantage of the sequence of the entire mitochondrial genome of *V. destructor* (Evans & Lopez 2002; Navajas *et al.* 2002) and the availability of published microsatellite markers (Evans 2000; Solignac *et al.* 2003) to provide a more precise picture of the genetic structure of *Varroa* populations. Our results are in agreement with previous work and show that the two *Varroa* types have a clonal origin and a very low variability within each type.

If this clonal structure of *Varroa* is well explained by a strong founder effect, the rarity of heterozygotes results from the reproduction system of the parasite. In the pseudo-arrhenotokous (Martin *et al.* 1997), haplo-diploid (de Ruijter & Papas 1983) mode of reproduction of *Varroa*, males result from fertilized eggs followed by subsequent elimination of the paternal complement (Sabelis & Nagelkerke 1992). The reproduction occurs in a capped comb cell where a honeybee larva (worker or drone) develops. A *Varroa* foundress enters the cell before operculation and lays successively a first egg giving a male, followed by several eggs (four or five, one every 30 h; Donzé & Guerin 1994), which develop into females. Development occurs in the operculated cell. The adult male copulates repeatedly with each of its sisters (adelphogamy). However, two, or more rarely several, foundresses may lay eggs in the same cell (Fuchs & Langenbach 1989; Martin 1995; Donzé *et al.* 1996). In that case, mating may occur between individuals of different lineages. Whereas high inbreeding is the rule, this behaviour might introduce genetic admixture.

The parasite presents 10–15 generations per year in temperate regions.

2. MATERIAL AND METHODS

(a) *Populations*

A total of 565 female mites belonging to 45 populations in 17 different countries with representatives from each continent were collected and analysed (table 1). A few individuals per population were chosen for genotyping. The quasi-clonal structure of the species justified this action, as it was futile to increase the numbers of individuals when the same multi-locus genotype being examined was present at high frequency (or even alone) in most populations. The high number of loci screened also compensated for the small number of individuals genotyped.

(b) *DNA extraction*

For microsatellite analysis and mtDNA typing, DNA was extracted from female mites by a Chelex method (Walsh *et al.* 1991), in a 100 µl volume adapted to the small size of the mites (*ca*. 1 mm length).

Polymerase chain reaction (PCR) amplifications for sequencing of mtDNA fragments used total DNA extracted by a cetyltrimethylammonium bromide (CTAB) procedure adapted for small organisms (Navajas *et al.* 1998).

(c) *Microsatellite markers*

We used 20 microsatellite loci: four among those published by Evans (2000) and 16 by Solignac *et al.* (2003). However, only 13 out of the 20 loci were retained for detailed analyses (see § 3).

Radioactive PCR conditions have been described in a primer note (Solignac *et al.* 2003). All variants were amplified and run again together with controls. A PCR of the DNA mini-preparation of the clone of the corresponding microsatellite was included in each run as a size marker, as well as a PCR of honeybee DNA as negative control.

(d) *Mitochondrial DNA*

(i) *Typing*

The mitochondrial haplotype may be assigned to one of the two *V. destructor* mtDNA types, using the restriction by *Sac*I of the PCR product of a fragment of the gene *CO-I* (Anderson & Fuchs 1998). A new primer pair was designed from the complete sequence of the mtDNA (Navajas *et al.* 2002): upper, 5′-TACAAAGAGGGAAGAAGCAGCC-3′; lower, 5′-GCCCCT ATTCTTAATACATAGTGAAAATG-3′. After a first denaturation step at 92 °C for 4 min, PCR was performed for 35 cycles of 1 min denaturation at 92 °C, 1.30 min annealing at 52 °C and 1.30 min extension at 72 °C. Restriction by the enzyme *Sac*I (2.5 U) of the 376 bp PCR product generated two fragments (128/124 and 252/256 bp) in Japan haplotypes, and a single (undigested) fragment in Korea haplotypes.

(ii) *Sequencing*

Specimens of *V. destructor* from 12 different origins and one *V. jacobsoni*, were analysed for mitochondrial sequences (table 1). Six fragments for a total of 5185 nucleotides were PCR amplified using primers (table 2) designed from the complete mitochondrial *V. destructor* sequence (Navajas *et al.* 2002). The 25 µl PCR reactions contained 2.5 µl of 10× *Taq* polymerase Qiagen buffer, 1 U of *Taq* polymerase Qiagen, 0.25 mM each dNTP, 0.5 µM each oligonucleotide primer, 5 µl of Q solution, 2.5 mM $MgCl_2$ and 2 µl of DNA sample. After a first denaturation step at 94 °C for 4 min, PCR was performed for 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at the appropriate temperature for each fragment (table 2) and 1 min extension at 72 °C. PCR products were directly sequenced using the BigDye Terminator method (Perkin Elmer, Foster City, CA, USA) in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems Inc.). PCR products were sequenced in both directions. Sequences were assembled using BioEdit v. 5.0.6 software (Hall 1999).

Table 1. The 45 populations analysed in this work for 13 microsatellite loci and mtDNA.
(*n*: number of individuals; *n* A: total number of alleles; K: number of individuals belonging to principal K type (100 100 for all loci); VK: number of the K type bearing variant allele(s); Hk: number of loci heterozygote for variant alleles in the K type; J: number of individuals belonging to the principal J type; VJ: number of individuals for variant alleles in the J type; Hj: number of loci heterozygote for variant alleles in the J type; F_1: number of F_1 hydrids K/J; KJ: number of individuals bearing J alleles at one or several loci (homo- or heterozygote); Hkj: number of loci heterozygote for a K and a J allele (F_1 hybrids not taken into account). mtDNA K: number of K restriction mitotypes; J: number of J mitotypes; seq: long sequence is available. Entries in bold: *Varroa* collected on *Apis cerana*. All others collected from *A. mellifera*.)

		microsatellites										mtDNA		
												PCR/*Sac*I		
sample	*n*	*n* A	K	VK	Hk	J	VJ	Hj	F_1	KJ	Hkj	K	J	seq
Avignon (S France)	92	17	85	7	1							17		1 K
Alsace (NE France)	13	14	12	1								13		1 K
Cévennes (S France)	12	13	12									2		
Ardèche (S France)	8	13	8									1		
Charente (SW France)	11	13	11									1		
Orne (NW France)	10	13	10									2		
Sarthe (NW France)	12	13	12									2		
Varsovie (Poland)	8	13	8									8		
Sheffield (England)	13	13	13									2		
Lockerbie (Scotland)	7	13	7									2		
Algeria	12	13	12									12		1 K
Pretoria (South Africa)	10	14	9	1	1							10		
Valdivia 1 (Chile A)	9	14	8	1	1							9		
Valdivia 2 (Chile B)	9	24	6			1			1	1		8	1	
Valdivia 3 (Chile C)	16	14	14	2								16		
La Union 1 (Chile D)	13	13	13									13		
La Union 2 (Chile E)	12	17	9							3	3	12		
Futrono 1 (Chile F)	14	20	5	$6+3^a$	2					3^a		14		
Futrono 2 (Chile G)	13	25	7	$2+1^a$					1	$2+1^a$	3	11	2	
Futrono 3 (Chile H)	14	15	6	$7+1^a$	2					1^a	1	14		
Santiago 1 (Chile I)	12	14	10	2	2							12		
Santiago 2 (Chile J)	23	13	23									23		
Argentina A	6	14	4	2								6		
Argentina B	7	13	7									7		
Sinnamary (French Guyana)	10	24	3			6			1			4	6	1 K
Mexico A (Mexico)	12	13	12									8		
Mexico B (Mexico)	13	13	13									6		
Mexico C (Mexico)	13	15	9	4								9		
Pennsylvania (USA)	6	15	5	1^b	1							6		
Michignan (USA)	15	18	9							6	5	15		1 K
Boulder Creek CA (USA)	11	15	10							1	2	11		1 J
Auckland (New Zealand)	6	14	5	1	1							6		
Bet Degan (Israel)	12	13	12									12		1 K
Bico, Luzon (Philippines)	9	13	9									9		1 K
Cebu City (Philippines)	8	15	5	3	2							8		1 K

(*Continued*.)

Table 1. (*Continued.*)

			microsatellites								mtDNA		
											PCR/*Sac*I		
sample	*n*	*n* A	K	VK	Hk	J	VJ	Hj	F_1	KJ	Hkj	K	J	seq
Beijing (China)	20	14	3	17	1							20		
Taichung (Taiwan)	12	16				2	10	1[c]					12	1 J
Yatsushiro (Japan)	2	14					2						2	
Yatsushiro (Japan)	19	14	18	1								19		
Machida (Japan)	7	25	1	5		1						6	1	
Machida (Japan)	15	27		10		2	2	1	1			10	5	
Noda (Japan)	7	25	1	3	1	1			1	1	1	5	2	
Yokohama (Japan)	1	13	1									1		
Tokyo (Japan)	6	14	2	4	1							6		1 K
Total	560		430	80 + 5[a]	16	13	14	2	5[d]	14 + 5[a]	15	378	31	
Kathmandu (Nepal)	5	24											5[e]	
Koya, Timur (Iran Jaya)	*Varroa jacobsoni*													JCBS

[a] Individuals bearing a Korea variant and some Japan alleles. [b] Individual bearing Korea variants at two loci. [c] Individual bearing one odd allele. [d] Heterozygote at all diagnostic loci (11 loci).
[e] Japan mtDNA type according to the rapid test.

(e) *Estimation of the proportion of double infestations*

Population samples in which pure types were found together with F_1 and other admixed individuals were used to estimate the proportion of infestation of the same cell by two *Varroa* females. We assumed that the probabilities of a single and a double infestation, noted p_s and p_d, respectively, add up to one. Let p_K, p_J and p_A be the proportions of pure Korea (K) and Japan (J) types and admixed females and α the proportion of F_1 issued from a cell infested by two females of different pure types. With proportion $(1-\alpha)$, these cells will produce pure type females. A pure K female is obtained either by a single infestation (Prob $= p_s$) by a pure K female (Prob $= p_K$) or by a double infestation (Prob $= p_d$) by two pure K females (Prob $= p_K p_R$) or by one K and one J female with proportion $(1-\alpha)/2$(Prob $= 2p_K p_J(1-\alpha)/2 = (1-\alpha)\, p_K p_J$). A similar rationale for the three other types of female leads to the following system:

$$\Pr(K) = p_s\, p_K + p_d\, (p_K^2 + (1-\alpha)\, p_K\, p_J),$$
$$\Pr(J) = p_s\, p_J + p_d\, (p_J^2 + (1-\alpha)\, p_K\, p_J),$$
$$\Pr(F_1) = p_d\, (2\alpha\, p_K\, p_J),$$
$$\Pr(A_1) = p_s\, p_H + p_d\, (p_H^2 + 2\, p_K\, p_H = 2\, p_J\, p_H).$$

To get posterior distributions of the proportion of double infestation marginal to all the other parameters, a Monte Carlo Markov Chain was created using uninformative priors and log-normal deviates for the proportions of the different types (p_K, p_J and p_A) and for parameters α and p_d. The likelihood was computed considering the sample as drawn from a multinomial distribution with parameters Pr(K), Pr(J), Pr(F_1) and Pr(A_1). Ten thousand values were sampled with a thinning of 1000 and a burn-in of 100 000 for each population sample. Distribution densities and statistics were obtained with the R-software (v. 1.7.1) using the *locfit* library.

3. RESULTS

(a) *Microsatellites*

(i) *Markers*

The 20 microsatellite loci examined showed very low variability (tables 1 and 3), except VJ275 which was too difficult to read and was discarded. Six additional loci were also excluded: four (VD109, VD115, VD134 and VD151) showed no variation at all on the first 400 genotyped individuals and two others (VD015 and VD121) showed a single and fixed variant in the Kathmandu sample. For the other 13 loci, 11 were diagnostic of the two types and the remaining two (VD146 and VD154) showed only intratype variability.

(ii) *Individuals*

The multilocus genotypes obtained for the 565 individuals of *V. destructor* can be classified into two categories, corresponding respectively to the Korea and Japan types, and infrequent individuals resulting from their hybridization.

The Korea type is by far the most abundant (510 individuals) and is present worldwide; 430 of them were homozygote for the *100* allele for all the 13 loci examined. Variants differ from allele *100* by the addition or subtraction of one, two or three dinucleotide motives (e.g. alleles *96*, *98*, *102*, *104* and *106*). These variants were present in the homozygote and more rarely in the heterozygote state

Table 2. Primer pairs used for PCR amplification of six regions of the mitochondrial DNA of *Varroa destructor*.
(Genes, product size (base pairs) and annealing temperature (T_a in degrees Celsius) are indicated. The nucleotide divergence in percentage is calculated between the K (10 samples) and the J (two samples) mitochondrial haplotypes of *V. destructor* and between *V. destructor* and *V. jacobsoni*.)

gene	primer name	primer sequences (5′–3′)	size (bp)	T_a	nucleotide divergence	
					between haplotypes (%)	between species (%)
cox1	10KbCOIF1	CTTGTAATCATAAGGATATTGGAAC	929	51	0.05	6.08
	6,5KbCOIR	AATACCAGTGGGAACCGC				
cox2–atp6	6,5KbCOII	GATTATTAGTTAGATCAGCAGACG	775	55	0.15	—
	6,5KbATP6	GTGTAAATACATAAGGTAATAACCC				
atp6–cox3	16KbATP6F	GACATATATCAGTAACAATGAG	818	51	1.03	5.60
	16KbCOIIIR	GACTCCAAGTAATAGTAAAACC				
nad3–nad5	6,5KbND3	CTATATCTTTTGAATGTGGATTTG	771	55	0.15	3.70
	6,5KbND5	CTGATTAGAATCATCTCTAACTCCCT				
cob	10KbCytbF-1	GCAGCTTTAGTGGATTTACCTAC	985	52	0.80	—
	10KbCytbPRIM	CTACAGGACACGATCCCAAG				
CR	10KbCRR7	CTGGACCAGACTCATATAG	327	50	0	1.24
	10KbCRF8	CATTTCCAATAAAGTCTAAGTG				

(electronic Appendix). They were very rare except allele *98* for VJ294 in Beijing (China), *102* for VJ292 in Chile and *104* for VD146 in Japan.

The Japan type is present in Japan, Taiwan, French Guyana and Chile. It differs from the preceding type at 11 diagnostic loci and the size of the principal allele differs from the one of the Korea type by any number of motifs between one and eight. Thirteen individuals belong to this type. Fourteen additional individuals (of which 10 came from Taiwan) bear a different allele at one or two of the 11 diagnostic loci. These variants show a range of allele size similar to the Korea type (differing by one or two motifs from the principal Japan allele). In several individuals from Taiwan there is a private allele at locus VD114 (allele *106*) which strikingly differs from the allele found in other Japan type individuals (allele *94*).

The few remaining individuals correspond to different categories. Five F_1 hybrids between Korea and Japan types have been observed (heterozygote K/J at the 11 diagnostic loci). Fourteen individuals with a general K profile carried J alleles at a limited number of loci and five additional ones had a K variant allele at another locus. Finally, five individuals from Kathmandu (Nepal) were strikingly distinct from all the others for the number and size of alleles and heterozygosity (electronic Appendix).

When the most frequent genotype (*100 100* for all loci) is discarded, the remaining ones over the 7345 (565 individuals $\times$ 13 loci) may be presented in a single table (electronic Appendix).

Allelic diversity at the 13 loci taken together is 2.00 for the K type, 1.31 for the J type, 3.08 combining the two, 1.92 for Kathmandu and 4.38 in the total sample.

(iii) *Populations*

They may be classified into four groups.

The vast majority of populations include only the K type and its variants. They are from all continents: Europe (all 10 populations studied), Africa (Algeria, Pretoria), the Americas (Argentina, five out of 10 samples from Chile, Mexico, Pennsylvania), Asia (Israel, China, Philippines, and Yatsushiro and Tokyo in Japan) and Oceania (New Zealand).

Only one population (Taiwan) included only the J type (Yatsushiro, collected on *A. cerana*, is a sample of only two individuals). For the other populations, the K type was also present in admixture with the J type (French Guyana, Chile, Japan). A single F_1 hybrid between the two clones was present in five populations (table 1). In these populations, recombinant individuals are very rare (one in Noda and four in Chile); they displayed a general K profile and some J alleles.

In some populations, the J clone is absent *per se* but its genetic signature at various loci may be observed. These segregating genotypes were observed for three populations in Chile and the USA. All the observed alleles differing from *100* have the same size as the J most frequent allele and are at low frequencies. Among the 19 introgressed individuals, 59 out of the 418 gene copies belonged to the J type, an average frequency of 0.14 (see figure 1 for the distribution). The two samples collected in Japan on *A. cerana* (from Yatsushiro on Kyushu Island and Machida on Honshu Island) show the same genetic profile (and the

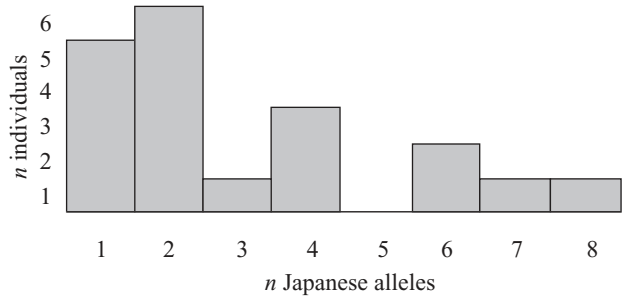

Figure 1. Distribution of the number of J alleles (counted one for heterozygote and two for homozygote) observed in K genetic background for the 11 diagnostic loci (22 gene copies).

same variant for VD126) as those collected from *A. mellifera* colonies. Their only peculiarity is the existence of a private variant, the allele *86* for VJ292.

(iv) *Heterozygosity*

Despite a low frequency, allelic variants are mostly homozygote. A total of only 94 heterozygotes has been observed among the 7345 (565 individuals × 13 loci) genotypes scored (*ca*. 1.3%): 16 of which correspond to the K genotype, two to a J genotype (with a doubt for allele *106* of VD114 in Taiwan), 70 between the two types (of which 55 for only the five F_1 hybrids at 11 diagnostic loci) and six for Kathmandu (12 loci only; this sample shows the highest heterozygosity, which reaches 8.6%).

(v) *Double infestation of cells by* Varroa *foundresses*

The low level of heterozygosity detected may be explained by autozygosity, which results from recurrent sib-mating. When heterozygotes are produced by crosses between two lineages in the case of two foundresses entering the cell (whether of the same type, but bearing different variants, or between the two types), their frequency does not drop immediately to zero but decreases rapidly in subsequent generations of brother × sister crosses. Similarly, neomutations may propagate in heterozygotes during several generations.

In this study, the sample sizes were too small to estimate the rate of double infestation from the genetic data within types. However, this information may be extracted from the rate of F_1 hybrids and recombinant between the two clones in the mixed populations exhibiting both the K and the J types (see § 2e).

Figure 2 provides the posterior distributions of the proportion of double infestation for the five samples containing F_1 individuals. These distributions are rather flat so that the modal values (French Guyana, 0.42; Machida, 0.32; Noda, 0.67; Chile B, 0.67; and Chile G, 0.92) should be considered with caution. The confidence intervals are very large but the 5% quartiles, ranging from 0.09 to 0.21, suggest that double infestations are frequent in the analysed samples.

(b) *Mitochondrial DNA*

(i) *Mitotypes*

Samples used for microsatellite analysis were typed using *Sac*I restriction of a PCR product of part of the *CO-I* gene; 414 individuals were analysed by this method. For the

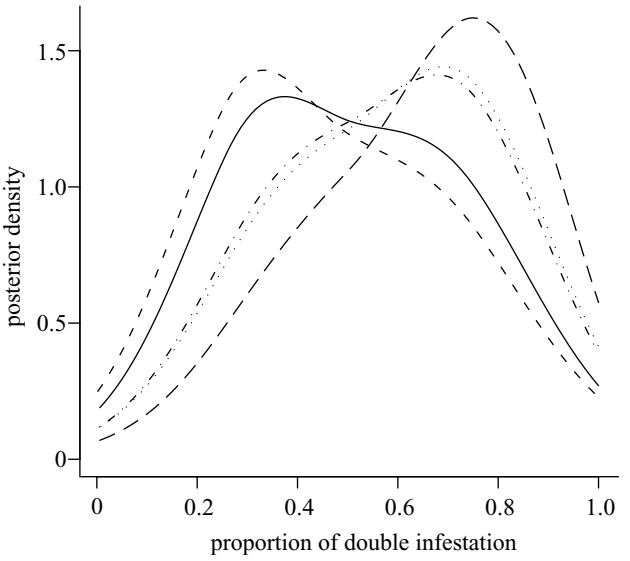

Figure 2. Posterior densities for the proportion of double infestation in five locations: French Guyana (solid line), Machida (small-dashed line), Noda (dotted line), Chile B (dash–dot line) and Chile G (long-dashed line). The coefficient α of the *locfit* command was set to 0.9.

European and Mexican populations, where only the K alleles were detected with microsatellites, 151 individuals (out of 211) were not studied. All individuals belonging to a pure K type showed the absence of the *Sac*I restriction site and all J nuclear types showed its presence. For the five F_1 hybrids, two carried a K mtDNA type and three a J type. Finally, among the 19 introgressed individuals, only one (Chile G) showed the J mitotype. The frequency of J nuclear alleles (0.14) is higher than the cytoplasmic one (0.06) but the difference is not significant (Fischer's exact test, $p = 0.49$).

(ii) *Sequences*

Mitochondrial sequences of the *CO-I* region of *V. destructor* individuals from 12 different origins showed that 10 samples belonged to the Korean haplotype and two to the Japan one (tables 1 and 2). The sequences of the six fragments spanning 5185 bp proved to be completely identical within each of the K and J types (table 2). Sequences of the K type are identical to the previously published complete sequence of the mitochondrial genome (*Varroa* from Avignon, France; EMBL accession number AJ493124; Navajas *et al.* (2002)). EMBL accession numbers for the sequences of the J haplotype are: (accession number: AJ784872). The two *V. destructor* haplotypes displayed a nucleotide divergence of 0.40% and they differ by 4.15% from *V. jacobsoni* (table 2). The lack of intratype mtDNA sequence variation is in total agreement with the microsatellite analysis.

The sequence of part (205 bp) of the *CO-I* mtDNA gene of individuals from the Kathmandu sample show that they belong to the Nepal haplotype of *V. destructor* collected on *A. cerana* and described by Anderson & Trueman (2000).

4. DISCUSSION

Varroa destructor is an invasive species that has dramatically expanded its geographical distribution after it shifted from its original host, *A. cerana*, to *A. mellifera*. Besides some

honeybee biological traits, such as drifting of workers and drones between colonies and swarming, anthropic factors like movements of frames, transhumance, displacement of colonies for pollination of fields and orchards, and the worldwide trade in live bees have played a major role in the rapid expansion of the parasite.

Invasive species frequently undergo founder effects that abolish most if not all of their genetic variability (Merilä *et al.* 1996; Tsutsui *et al.* 2000). This also appears to be the case for *Varroa* and this is the reason why we have, wherever possible, used microsatellite loci and sequenced a large part (approximately one-third) of the mtDNA, although further studies are needed to determine the levels of variation of these mites on *A. cerana* in the Korea/Japan/China region.

Although previous studies have detected the major bipartition of this new parasite of the Western honeybee (see § 1 for references), we provide an important number of resolutive markers that can easily distinguish between the Korea and Japan types and establish the genotypic structure of all individuals (co-dominance). In addition, their multiplicity allows detection of low levels of introgression. They also detected a nascent intratype variability in populations, which provides some information on the biology and the origin of the parasite.

(a) *Clonal origins of the parasite*

The very low variability detected is not attributable to structural or functional reasons resulting in a lower mutation rate of microsatellites in *Varroa*. The markers are actually variable in *V. jacobsoni* (Solignac *et al.* 2003), in the Kathmandu sample, and between the K and J types. Similarly, the mitochondrial sequences are also variable between the two types and different from *V. jacobsoni*.

The reduced variability may rather be the result of a severe bottleneck, which probably occurred at the time of the parasite transfer from *A. cerana* to *A. mellifera*. It is not posterior to this transfer as shown by the absence of a structure of populations. In addition, the dramatic increase of population size would have prevented the erosion of the variability.

Founder events have happened for the two types of *V. destructor*, the Japan type and the Korea type. These transfers were probably independent, occurred at different times, and from different populations (although from a small region of Asia) of *Varroa* parasitizing their natural host *A. cerana*. Several facts support this hypothesis: the historical data, the different microsatellite profiles, their almost complete linkage disequilibrium, the differences in mitochondrial sequences and the reproductive isolation between the clones in three countries (see § 4h). Then, the 'biclonal' structure observed in Japan is not the original one (from which the two clones would have emerged) but the result of a secondary admixture, as in other countries.

The mtDNA sequences show that the *Varroa* population from Kathmandu on *A. mellifera* is related to a third type of *V. destructor* described from *A. cerana* (Nepal haplotype; Anderson & Trueman 2000). It is not known if these mites reproduce on *A. mellifera*.

(b) *Origin of the intraclone variability*

The allelic differences between the two clones consist of a variable number of repeated motifs, from zero to eight. The intraclonal variability has a strikingly different spectrum: variant alleles differ from the principal one by one (10 cases), two (five cases) or three (one case) repeated motifs (electronic Appendix). These characteristics, associated with the very low frequency of almost all variant alleles and the fact that they are mostly private indicates that mutations appeared subsequently to the clone foundations. At the very origin of the parasitism of the Western honeybee, all the individuals should have had one of the two genetic formulae indicated in the upper part of the electronic Appendix. The alleles differing from the principal allele by two or three motifs probably arose in a single step because the hypothetical intermediate one-step alleles are either absent or very rare in the whole sample. The single case of an odd-sized allele is *106* for VD114 in Taiwan (the J most frequent allele is *94*), which could result from an infrequent mutation or have an origin external to the clone.

It cannot be ruled out that the same mutation occurred twice: allele *98* for the marker VD154 in Mexico and Philippines, *102* for marker VJ292 in France, Chile, Philippines, *96* for marker VJ295 in France and New Zealand, *102* for the same marker in South Africa, Argentina and Pennsylvania. Alternatively, they may have a common origin and be propagated by intercontinental movements.

(c) *Pattern of mutation*

Microsatellite pattern evolution has been reviewed by Estoup & Angers (1998). The simplest model is the stepwise mutation model (SMM) in which mutations add or remove a single repeat to the microsatellite core sequence (Valdes *et al.* 1993). Since then, slightly more complex models have been suggested such as the two phase model (TPM; DiRienzo *et al.* 1994) or the generalized stepwise model (GSM; Fu & Chakraborty 1998) which allow for multiple repeat mutations. Whittaker *et al.* (2003) considered more refined models, which assume mutation rates vary with the number of repeats of alleles. Our observations of only 16 mutations cannot be used in a complex model that includes many parameters. However, considering their simplest model, equivalent to the GSM, a simple calculation indicates that the estimate of the parameter of the geometric distribution (p, formula (1) in Fu & Chakraborty (1998)) found in our data ($p = 0.304$) is close to that of Whittaker *et al.* ($p = 0.346$) and well within their 95% confidence interval (0.239–0.502). This confirms that the probability of mutations involving more than a single repeat is far from negligible.

(d) *Rate of mutation and time of separation of the Korea and Japan lineages*

It is rather difficult to estimate from simple observation of the current genetic structure if the K and J clones are derived from a single population or if they originated from different populations. Historical data, mtDNA signatures and postzygotic isolation (see § 4h) suggest that the later hypothesis is probably correct.

Considering that mutations within each type have occurred in the past 50 years, and that the two types have different alleles at many loci, our data allow estimation of the divergence time of the two types. An analysis based on Monte Carlo Markov chains (J.-M. Cornuet, M. A. Beaumont, A. Estoup and M. Solignac, unpublished data)

was performed to provide an estimate of this time of divergence. For a large set of parameters, estimates fall in an interval of 5000–15 000 years. The same analysis also provides posterior distributions for mutations rates. The medians range from 2×10^{-5} to 3.4×10^{-4} for polymorphic loci and are *ca*. 1.6×10^{-5} for monomorphic loci; these figures are well within the range of that of other species (Estoup & Angers 1998).

(e) *Relationships between the two clones*

Molecular results obtained in this work are in general agreement with data reported in the literature. The first RAPD analyses by de Guzman *et al.* (1997, 1999) detected only one type in each country or area studied except in the United States and Canada. Using microsatellite markers, we show that the distribution of the two types is more complex. In particular, if populations with their Russian type (= the Korea type) only are numerous, we have found only a single population comprising the J type alone (Taiwan) contrary to these authors (Japan, Puerto Rico, Brazil). Several explanations could account for these different results: (i) the use of more resolutive markers; (ii) the analysis of different sampling localities; (iii) a change in the populations of parasites since their study. We suspect that the last explanation is the correct one; it implies that the K type, far more virulent, is displacing the J type in many countries.

(f) *Heterozygosity, double foundations*

Only 94 cases of heterozygosity have been observed among the 7345 scored genotypes. This observation may be related to the low frequency of variant alleles but also to the mite reproductive behaviour (adelphogamy). Heterozygotes can be generated in two different ways: either because a neo-mutation occurred in the ancestry of the heterozygote or because a cross occurred between variants within types or between the two types. Under strict adelphogamy, neo-mutations are quickly eliminated or fixed. In areas where the two types coexist, the presence of admixed genotypes (including F_1) in several samples indicates that double infestations are not rare. Our estimates, although imprecise, suggest that the proportion of double infestations is noticeably higher than that resulting from empirical data on brood cells (Fuchs & Langenbach 1989; Martin 1995; Donzé *et al.* 1996). However, these values are the result of diverse parameters, mainly the level of infection, survival of individuals, sex ratio and density-dependent fecundity. Because no evidence exists for an aggregative behaviour for *Varroa* females when invading the cells, a higher rate of double infestation should be related to a higher level of parasitism (Salvy *et al.* 1999).

(g) *Japanese alleles in recombinant individuals*

The number of J alleles introgressing K genotypes varies between one and eight for 22 opportunities per genome (11 diagnostic loci). This distribution suggests that this is the only direction of introgression present in our samples. In the inbred progeny of F_1 hybrids, reproducing only by adelphogamy, 11 J alleles (5.5 homozygote loci) are expected. An average of only 3.11 J alleles were observed for individuals bearing them. This bias requires approximately two backcrosses of hybrids with the dominant K clone and hence double infestations and backcrosses. Although backcrosses to the frequent K type are the most probable, and this suffices to explain this situation, a selection against the J alleles is also possible.

(h) *Taxonomic status of the two clones*

Sexual isolation is probably absent between the two clones in the case of admixture (in the reverse case, the above calculation of double infestation should be even higher). These data suggest that, for these populations, F_1 hybrids are recurrently produced at a rate of *ca*. 10% per generation (five F_1 hybrids among 54 individuals). It is therefore difficult to explain why these populations are not entirely composed of segregants without invoking a strong post-zygotic isolation between the two types. Alternatively, a recent admixture might also account for these results. However, it should be so recent, occurring simultaneously in several remote localities (in Japan, French Guyana and Chile) and on the two host species (in Japan), that this hypothesis is unlikely.

The post-zygotic isolation is, however, not complete and seems heterogeneous in different populations. In populations showing a mixture of the two clones, segregants are either entirely absent or very rare (zero in French Guyana and Machida, one in Noda and Chile B and two in Chile G). In other populations (presence of J alleles but not of the J type *per se*), the segregants might be more numerous (one for Chile F and H, three for Chile E and six for Michigan). This heterogeneity is surprising because the contact is always between the same two clones.

(i) Apis cerana–A. mellifera *back and forth*

de Guzman *et al.* (1997) deduced, using RAPD markers, the identity of the parasites of *A. mellifera* and *A. cerana* in Japan. In the present study, a similar result was obtained using microsatellites. Our results show that the parasites of the two host species share the same polymorphism. The only way to explain these data is that one species received the parasite from the other. Because the parasites of *A. mellifera* are the same in all populations examined (although those of *A. cerana* are differentiated at least for mtDNA; Anderson & Trueman 2000), in Japan *A. cerana* appears to be the recipient species from the local *A. mellifera*. It is thus highly probable that after a first host transfer of *Varroa* from *A. cerana* to *A. mellifera*, the parasite shifted back from *A. mellifera* to *A. cerana*.

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