Apidologie 41 (2010) 181–193
© INRA/DIB-AGIB/EDP Sciences, 2009
DOI: 10.1051/apido/2009068

Available online at:
www.apidologie.org

Original article

New Asian types of *Varroa destructor:* a potential new threat for world apiculture*

Maria NAVAJAS[1], Denis L. ANDERSON[2], Lilia I. de GUZMAN[3], Zachary Y. HUANG[4], Jeremy CLEMENT[1], Ting ZHOU[5], Yves Le CONTE[6]

[1] INRA, UMR CBGP (INRA/IRD/Cirad/Montpellier SupAgro), Campus International de Baillarguet, CS 30016, 34988 Montferrier-sur-Lez Cedex, France
[2] CSIRO Entomology, PO Box 1700, Canberra, ACT 2601, Australia
[3] USDA/ARS, Honey-Bee Breeding, Genetics and Physiology Laboratory, 1157 Ben Hur Road, Baton Rouge, LA 70820, USA
[4] Department of Entomology, Michigan State University, East Lansing, MI 48824, USA
[5] Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, China
[6] INRA, UMR INRA/UAPV Abeilles et Environnement, Laboratoire Biologie et Protection de l'Abeille, Site Agroparc, Domaine Saint-Paul, 84914 Avignon Cedex 9, France

Received 22 January 2009 – Revised 17 July 2009 – Accepted 21 August 2009

Abstract – The invasion of the Western honey bee, *Apis mellifera,* by *Varroa destructor* is attributed to two mitochondrial haplotypes (K and J) that shifted last century from their primary host the Eastern honey bee, *A. cerana,* in north-east Asia. Here, mitochondrial DNA sequences (*cox1*, *cox3*, *atp6* and *cytb*: 2700 base pairs) were obtained from mites infesting both Eastern and Western honeybees (respectively 21 and 11 colonies) from Asia including regions where the shifts first occurred. A total of eighteen haplotypes were uncovered in Asia (11 on *A. cerana* and 7 on *A. mellifera*). Two new variants of the K haplotype and two of the J haplotype were found on Western honeybees in what appeared to be well-established infestations. New haplotypes may represent a potential threat to *A. mellifera* worldwide. The extreme lack of polymorphism in the K and J haplotypes outside of Asia, can now be plausibly explained as being due to genetic 'bottlenecks' that occurred in Asia before and after mites shifted from their original Eastern honeybee host.

***Apis mellifera* / *Apis cerana* / *Varroa* / mitochondrial DNA/diversity**

1. INTRODUCTION

The varroa mite, *Varroa destructor* (Acari: Varroidae), is a well-adapted parasite of the Eastern honeybee (*Apis cerana*) in northern regions of mainland Asia. It also dramatically expanded its distribution when it shifted host to the Western honeybee (*A. mellifera*) in the last century. Prior to and long after the shift, *V. destructor* was mistaken for *V. jacobsoni*, a morphologically similar species first described from *A. cerana* in Indonesia (Oudemans, 1904). It was not until 2000, following a comprehensive molecular and morphological studies of so-called *V. jacobsoni* mites on *A. cerana* throughout Asia, that it was recognized as a distinct species (Anderson and Trueman, 2000). In that particular study, 18 different mite haplotypes [mites with distinct mitochondrial (mt) DNA sequences] were discovered, nine of which were members of *V. jacobsoni*, six of *V. destructor*, and the identities of three others were unresolved. Each haplotype was named after the country or island in which it was first found, e.g., the China 1 (C1) haplotype was found in China. One more haplotype of *V. destructor* has subsequently been found on *A. cerana* in China

Corresponding author: M. Navajas,
navajas@supagro.inra.fr
* Manuscript editor: Klaus Hartfelder

using the same marker (China 2 or C2) (Zhou et al., 2004).

Of the eight known haplotypes of *V. destructor* on *A. cerana*, only Japan 1 (J1) and Korea 1 (K1) have successfully colonized *A. mellifera* (Anderson and Trueman, 2000; Solignac et al., 2005). J1 first shifted from *A. cerana* to *A. mellifera* in Japan last century following the introduction of *A. mellifera* (Sakai and Okada, 1973). It then spread on *A. mellifera* into Thailand and Brazil (Anderson and Trueman, 2000) and then into North America (de Guzman et al., 1999). According to de Guzman et al. (1997) and Oldroyd (1999), K1 first switched from *A. cerana* to *A. mellifera* near Vladivostok (north of the Korean Peninsula) following the introduction of *A. mellifera* from Ukraine during the late 1950s (Crane, 1978). It thereafter spread on *A. mellifera* into Europe in the 1960s (Crane, 1978), then around the world, arriving in North America in 1987 (de Guzman et al., 1997; Oldroyd, 1999; Sammataro et al., 2000). On *A. mellifera*, J1 and K1 are partially isolated clones (Solignac et al., 2005) for reasons that are not understood.

Here, we sought to gain further insights into the invasion of *A. mellifera* by *V. destructor* by identifying and genotyping isolates of *V. destructor* infesting both *A. cerana* and *A. mellifera* in regions where the J1 and K1 host shifts first occurred as well as in a broader area of *V. destructor*'s natural geographic range in Asia. The aim of our study was to obtain insights into the following questions: (i) how variable is *V. destructor* on its primary host (*A. cerana*) and its new host (*A. mellifera*) in Asia where the J1 and K1 host shifts occurred? and (ii) are J1 and K1 on *A. mellifera* derived from different mite populations, or do they simply reflect haplotypes randomly sampled in a single large *Varroa* population?

2. MATERIALS AND METHODS

Female *Varroa* mites were collected from 21 *A. cerana* and 11 *A. mellifera* colonies in several Asian countries where *V. destructor* is known to occur alone (China, Japan, Korea, far East Russia, Taiwan and North Vietnam) or sympatric with *V. jacobsoni* (Thailand) (Tab. I). Several mites per colony were collected alive from the bee brood and preserved in 95% ethanol until needed for DNA analysis. In most cases, a single bee colony was sampled at one locality, the exceptions being where *A. mellifera* and *A. cerana* colonies were hived next to each other in the same apiary, as in Nanchang (China), where 8 colonies were sampled (5 *A. cerana* and 3 *A. mellifera*), or where *V. destructor* and *V. jacobsoni* exist sympatrically as in Chang Mai, Thailand where 5 colonies were sampled. In all cases, three mites were molecularly analyzed from each colony sampled, except in the case where 10 mites were analyzed from a single *A. mellifera* colony from Chang Mai (Thailand).

Total DNA was extracted from single mites using the DNeasy tissue Kit (Qiagen, USA), following manufacturer's instructions as indicated for the spin-column protocol from Cultured Animal Cells with the following slight modifications: (i) all volumes were reduced to half; (ii) incubation was at 70 °C for 3 h; (iii) final elution was in 50 μL of DNase-free water. Four fragments from either the *cox1*, cytochrome oxidase III (*cox3*), ATP synthase 6 *(atp6)* and cytochrome b (*cytb)* mitochondrial genes were amplified by the polymerase chain reaction (PCR) from each *V. destructor* mite whereas only fragments of the *cox1, cox3, atp6* genes (same sequences as above), were amplified from *V. jacobsoni* mites (Tab. I). The PCR primers (Tab. II) were developed from published sequence of the complete mtDNA genome of *V. destructor* (GenBank Accession No. AJ493124.1; Evans and Lopez, 2002; Navajas et al., 2002). A 458 base pair (bp) fragment of the *cox1* gene was also amplified for each mite using C1/C1R primers defined on sequences published by (Anderson and Fuchs, 1998). Reactions were carried out in 25 μL of PCR solution containing 2.5 μL of 10X buffer, 1U Taq polymerase (Qiagen), 0.25 mM of each dNTP, 0.5 μM of each oligonucleotide primer, 2.5 mM of $MgCl_2$ and 2 μL of template DNA. Samples were denatured at 94 °C for 4 min and then PCR was done for 35 cycles of 30 s denaturation at 94 °C, 30 s annealing (see Tab. II for annealing temperature for each fragment) and 1 min extension at 72 °C. PCR products were cleaned-up using the ExoSAP-IT® reagent and subsequently directly sequenced in two directions using the BigDye® Terminator method (Parkin Elmer, Foster City, CA, USA) in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems Inc). Sequences obtained using the C1/C1R primers were used to assign mites to a particular *cox1* haplotype for direct comparison with haplotypes found

Table I. Origin, collection details and identity of *Varroa destructor* (VD) and *V. jacobsoni* (VJ) isolates examined from *Apis cerana* (Ac) and *Apis mellifera* (*Am*) (shaded) collected in Asia. Mites were assigned to a known (e.g. V1) or new haplogroups based on partial nucleotide sequence (458 bp) of their mtDNA *cox1* gene (Anderson and Trueman, 2000). Haplotypes were also further defined (e.g. V1-2) based on concatenated nucleotide sequences of fragments of their mtDNA *cox1*, *cox3*, *atp6* and *cytb* gene sequences (2700 bp) obtained in this study.

Country	Locality	Mite species	Bee host	Collection year	Collector	Colonies examined	Mite MtDNA *cox1* Haplotype[1]	Concatenated mtDNA haplotype	GenBank accession numbers			
									cox1	*atp6*	*cox3*	*cytb*
China	Kunming, Yunnan Prov.	VD	*Ac*	2002	D. L. Anderson	1	C2	C2-1	GQ379067	GQ379124	GQ379086	GQ379105
China	Dayao Co. Yunnan Prov.	VD	*Ac*	2002	Z. Huang	1	C3 (new)	C3-1	GQ379068	GQ379125	GQ379087	GQ379106
China	Xishuanbanna, Yunnan Prov.	VD	*Am*	2002	Z. Huang	1	K1	K1-4	GQ379060	GQ379117	GQ379079	GQ379098
China	Xishuanbanna, Yunnan Prov.	VD	*Ac*	2002	Z. Huang	1	V1	V1-2	GQ379062	GQ379119	GQ379081	GQ379100
China	Zhuhai, Guangdong Prov.	VD	*Ac*	2001	Z. Huang	1	C1	C1-1	GQ379065	GQ379122	GQ379084	GQ379103
China	Zhongshan, Guangdong	VD	*Ac*	2002	Z. Huang	1	C1	C1-2	GQ379066	GQ379123	GQ379085	GQ379104
China	Nanchang, Jiangxi Prov.	VD	*Ac*	2004	Y. Le Conte	5	K1	K1-3	*	*	*	*
China	Nanchang, Jiangxi Prov.	VD	*Am*	2004	Y. Le Conte	3	K1	K1-2	GQ379057	GQ379114	GQ379076	GQ379095
China	Hunan Prov.	VD	*Ac*	2004	Y. Le Conte	1	K1	K1-3	GQ379059	GQ379116	GQ379078	GQ379097
Japan	Tokyo (Tamagawa Uni)	VD	*Ac*	1994	D. L. Anderson	1	J1	J1-2	GQ379070	GQ379127	GQ379089	GQ379108
Japan	Tokyo	VD	*Am*	1996	T. Yoshida	1	J1	J1-6	GQ379074	GQ379131	GQ379093	GQ379112
Japan	Tokyo[3]	VD	*Am*	2000	T. Gotoh	1	K1	K1-1	*	*	*	*
Japan	Machida	VD	*Ac*	1998	A. Sylvester	1	J1	J1-3	GQ379071	GQ379128	GQ379090	GQ379109
Japan	Shikoku	VD	*Ac*	1996	T. Yoshida	1	J1	J1-4	GQ379072	GQ379129	GQ379091	GQ379110
Korea	Seoul	VD	*Am*	1996	T. Stelzer	1	K1	K1-1	GQ379056	GQ379113	GQ379075	GQ379094
Russia	Vladivostok	VD	*Am*	1995	D. L. Anderson	1	K1	K1-1	*	*	*	*
Taiwan	Taichung[3]	VD	*Am*	2002	Chyi-Chen Ho	1	J1	J1-1	GQ379069	GQ379126	GQ379088	GQ379107
Thailand	Bang Changtay	VD	*Ac*	2003	M. Navajas	1	V1	V1-4	GQ379064	GQ379121	GQ379083	GQ379102
Thailand	Bang Changtay	VJ[2]	*Ac*	2003	M. Navajas	1	L1	L1-1	GQ387678	GQ387676	GQ387674	-
Thailand	Chiang Mai	VJ[2]	*Ac*	2003	M. Navajas	2	L1	L1-2	GQ387679	GQ387677	GQ387675	-
Thaïland	Chiang Mai	VD	*Am*	1997	L. de Guzman	1	J1	J1-5	GQ379073	GQ379130	GQ379092	GQ379111
Thaïland	Chiang Mai	VD	*Ac*	2003	M. Navajas	2	V1	V1-3	GQ379063	GQ379120	GQ379082	GQ379101
Vietnam	Hanoi	VD	*Ac*	1996	L. de Guzman	1	V1	V1-1	GQ379061	GQ379118	GQ379080	GQ379099
Vietnam	Hanoi	VD	*Am*	1996	L. de Guzman	1	K1	K1-2	GQ379058	GQ379115	GQ379077	GQ379096

* Sequences identical as other samples having the same haplotype.

[1] Haplotype names have been abbreviated: China 1 (C1), China 2 (C2), China 3 (C3), Japan 1(J1), Vietnam 1 (V1), Korea 1 (K1), Laos 1 (L1).

[2] Haplotype determined on basis of 1635 bp fragments of the mtDNA *cox1* , *cox3* and *atp6* genes.

[3] Mites issued from the same population used in Solignac et al. (2005). The obtained sequences are used here for haplotype correspondence with the previous K1-1 (Tokyo) and J1-1 (Taichung) haplotypes found on *A. mellifera.*

Table II. DNA amplified fragments to study variation in *Varroa destructor* samples. Amplified gene fragment, product size base pairs (bp) and annealing temperatures (Ta) are indicated. PCR primers defined based on the complete mitochondrial genome sequence of *V. destructor* (Navajas et al., 2002).

Fragment	Primer name	Primer sequences (5'-3')	Size (bp)	Ta (°C)
cox1	10KbCOIF1	CTT GTA ATC ATA AGG ATA TTG GAAC	929	51
	6,5KbCOIR	AAT ACC AGT GGG AAC CGC		
atp6-cox3	16KbATP6F	GAC ATA TAT CAG TAA CAA TGAG	818	51
	16KbCOIIIR	GAC TCC AAG TAA TAG TAA AACC		
cytb	10KbCytbF-1	GCA GCT TTA GTG GAT TTA CCT AC	985	52
	10KbCytbPRIM	CTA CAG GAC ACG ATC CCA AG		
*cox1**	C1	GCG GTT CCC ACT GGT ATT	458	52
	C1R	AAT ACC AGT GGG AAC CGC		

*Fragment partially included in the 929 nucleotides long *cox1* sequence and used here to sort out samples according to the published haplotypes (Anderson and Trueman, 2000).

in previous studies (Anderson, 2000; Zhou et al., 2004; Solignac et al., 2005), whereas sequences obtained using the other primers allowed the detection of variation within the haplotypes defined using cytochrome oxidase I (*cox1)* sequences (458 bp).

Sequences were assembled using the STADEN package 1.6 (http://staden.sourceforge.net/) and edited with BioEdit V.5.0.6 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Sequence alignments were initially performed using ClustalW version 1.8 (Thompson et al., 1994) and optimized by visual inspection. Sequences obtained from individual mites were concatenated into a single data set to increase the power of haplotype relatedness reconstruction. Concatenated sequences of the *cox1, cox3, atp6* and *cytb* genes of each *V. destructor* haplotype (2700 nucleotides) was aligned by eye without the need for numerical algorithms. Concatenated sequences of fragments of the *cox1, cox3* and *atp6* genes from *V. jacobsoni* haplotype contained 1635 nucleotides (*cytb* PCR primers failed to amplify *V. jacobsoni* DNA). Identical sequences were grouped and defined as one haplotype. Standard sequence diversity indices (Nei, 1987) were computed including *A* (number of alleles = variable haplotypes), *S* (number of segregating sites = variable nucleotide positions), and π (nucleotide diversity) using the DnaSP 4.10.4 package (http://www.ub.es/dnasp/) (Rozas et al., 2003). Intraspecific haplotype genealogies based on the 2700 bp *V. destructor* sequences were reconstructed. Gene genealogies were first inferred by computing haplotype network methods using the TCS program v2.1 (Clement et al., 2000), which essentially gave the same information as a phylogenetic reconstruction based on the Neighbour-Joining methods as implemented in the MEGA Package version 3.1 (Kumar et al., 2004). Only the resulting phylogenetic tree is presented here.

3. RESULTS

3.1. Mite variation based on a 458 bp fragment of the mitochondrial *cox1* gene

Seven different sequences were identified among DNA fragments amplified from a total of 103 *Varroa* mites collected from 32 colonies using the previously defined C1/C1R primers. Five sequences were identical to either the published China 1 (C1), China 2 (C2), Japan 1 (J1), Korea 1 (K1) or Vietnam 1 (V1) haplotypes of *V. destructor* (Anderson and Trueman, 2000; Zhou et al., 2004) (GenBank accession numbers: AF106900, AY372063, AF106897, AF106899 and AF106901, respectively). A sixth sequence was identical to a Laos 1 (L1) haplotype of *V. jacobsoni*, which was first described by Zhou et al. (2004). A new sequence was also identified and is hereafter referred to as the China 3 (C3) haplotype of *V. destructor*. Only *V. destructor* haplotypes were detected in geographical regions (China, Japan, Korea, Russia, Taiwan and Northern Vietnam) where *V. destructor* had been previously found

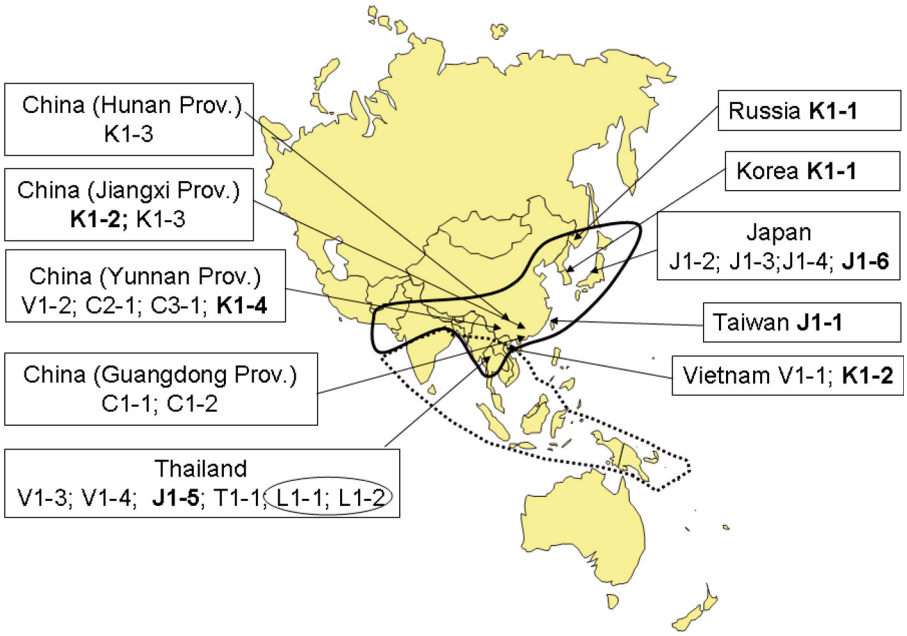

Figure 1. Geographical distribution of *Varroa destructor* mitochondrial haplotypes on *Apis cerana* and *A. mellifera* (in bold). All haplotypes were identified as *V. destructor* except two L1 haplotypes, which belong to *V. jacobsoni* (circled) from Thailand. The *V. destructor* haplotypes found on *A. mellifera* elsewhere are K1-1 and J1-1 (Solignac et al., 2005). Solid line indicates approximate range of *V. destructor* and dotted line is that of *V. jacobsoni*, after Anderson and Trueman (2000) and data provided by D.L. Anderson.

to occur alone (Tab. I, Fig. 1). Both *V. destructor* and *V. jacobsoni* haplotypes were found in Thailand where both mites had been previously found sympatrically (Fig. 1). In all cases, the three mites studied per colony had identical mitochondrial sequences as did the 10 mites analyzed from an *A. mellifera* colony near Chang Mai (Thailand).

3.2. Mite variation based on concatenated mtDNA sequences

The concatenated sequences revealed greater genetic variation among mites (18 haplotypes; Tab. III) than was revealed by the sequences of the 458 bp *cox1* fragment alone (7 haplotypes, see previous section). Hence, mites with identical *cox1* sequences were regarded as members of the same 'haplogroup', of which there were 7 and, mites of the same haplogroup that showed variation within their concatenated sequences were regarded as variants of a particular haplogroup.

Among mites collected from *A. mellifera* in regions of northeast Asia where only *V. destructor*, not *V. jacobsoni*, has been previously reported, 3 variants were found of the K1 haplogroup (K1-1, K1-2, and K1-4) and 2 variants of the J1 haplogroup (J1-1 and J1-6). Among mites collected from *A. cerana* in this same region, 1 variant was found of the K1 haplogroup (K1-3), 3 of the J1 haplogroup (J1-2, J1-3 and J1-4), 2 of the V1 haplogroup (V1-1 and V1-2) and 1 variant each of the C2 and C3 haplogroups (C2-1 and C3-1). Hence, only variants of *V. destructor* were found on *A. mellifera* and *A. cerana* in these regions. Also, only variants of the K1 and J1 haplogroups were found on *A. mellifera* and none of those variants were found on *A. cerana*. Among mites collected from Thailand, where *V. destructor* and *V. jacobsoni* coexist, two variants were found of the V1 haplogroup on *A. cerana* (V1-3 and V1-4) and two of the

Table III. Nucleotide positions of aligned mitochondrial sequences of four genes (*cox1*, *atp6*, *cox3* and *cytb*) that distinguish haplotypes of *Varroa destructor*. Dots indicate identical nucleotide as in the first sequence. Figures correspond to position numbers in the complete mitochondrial sequence of *V. destructor* (AJ493124.1). Mite samples were collected on either *Apis mellifera* (shaded haplotype code) or *Apis cerana*.

Haplotype	Locality (country)	Variable sites: *cox1*	*atp6*	*cox3*	*cytb*
					1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
		1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2	4 4 4 4 4 4 4 4 4	4 4 4 4 4 4 4 4 4 4 4 4 4	9 9 9 9 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
		4 4 4 4 4 6 6 6 6 7 7 7 8 8 8 8 9 0 0 1 2 3	0 1 1 1 1 2 2 3 4	5 5 5 5 5 5 5 6 6 7 7 7 7	8 8 9 9 0 0 0 0 1 1 2 3 4 4 4 4 5 5 5 6 7
		2 3 7 7 9 3 5 5 7 1 4 8 0 0 4 6 7 3 5 9 0 1	9 4 6 6 7 5 5 1 0	0 3 3 4 4 5 9 3 6 0 1 6 7	5 7 6 9 2 4 6 7 5 9 7 3 3 5 7 9 3 8 9 3 1
		2 4 3 6 7 8 6 9 4 6 9 8 3 9 5 3 4 1 5 9 2 6	7 5 0 3 6 0 9 3 0	7 7 8 7 9 7 1 9 7 8 1 2 8	5 0 1 6 3 1 2 1 5 7 2 6 4 0 6 5 9 1 9 2 3
K1-1	Seoul (Korea)	TGTATTCCTGGGATATACGCGA	AGACTGGTT	ACTTATCATACCT	ACAGTCTTGGCTCAATCACTT
K1-2	Hanoi (Vietnam)	....	....	....	....
K1-3	Hunan (China)	............G..A......	....	....	....
K1-4	Xishuanbanna China)	.....................G	....	....	....
V1-1	Hanoi (Vietnam)	..C.....A..AGC.AG.A...	GA......C	..CCT.T..G.TA	..GA.T..AAT.......T.C
V1-2	Xishuanbanna (China)	..C.....A..AGC.AG.A...	G.......C	..C.T.T.CG.TA	.TGAAT.......G.C..T.C
V1-3	Chiang Mai (Thailand)	..C.....A..AGC.AG.A...	G.......C	..C.T.T.CG.TA	.TG..T..A........GT.C
V1-4	BangChangtay (Thailand)	..C.....A..AG..AG.A...	G.......C	....T.T..G.T.	.TGAAT..A......C..T.C
C1-1	Zhuhai (China)	..CG.CT.A..AG..AGTA.A.	GA...T..C	.TC.TCT..G.T.	.TG..T..A........GT.C
C1-2	Zhongshan (China)	..C..CT.A..AG..AGTA.A.	GA...T..C	..CCTCT..G.T.	.TG..T..A........GT.C
C2-1	Kunming (China)	CAC...TT.AA.G.GA..A...	...TC...C	.TC.T.T..GTT.	..G..TGAA..CT.....T.C
C3-1	Dayao (China)	C.C....T.AA.G..A.TA..G	...T....C	..C.T.T..GTT.	..G..TG.A...T.....T.C
J1-1	Taichung (Taiwan)	....C.......G..A..AT..	...T..AA.	G.....TG...T.	GT...T..A.....G.T.TC.
J1-2	Tokyo (Japan)	....C.......G..A..AT..	...T..AA.	G.....TG...T.	.T...T..A.....G.T.TC.
J1-3	Machida (Japan)	....C.......G..A..AT..	..GT..AA.	G.....TG...T.	.T...T..A.......T.TC.
J1-4	Shikoku (Japan)	....C.......G..A..AT..	..GT..AA.	G.....TG...T.	.A...T..A.......T.TC.
J1-5	Chanm Mai (Japan)	....C.......G..A..AT..	...T..AA.	G.....TG...T.	.TGAAT..A......C..T.C
J1-6	Tokyo (Japan)	....C.......G..A..AT..	...T..AA.	G.....TG...T.	.A...T..A.....G.T.TC.

L1 haplogroup (L1-1 and L1-2) also on *A. cerana*. A variant of the J1 haplogroup (J1-5) was found on *A. mellifera*. Hence, only *V. destructor* was found on *A. mellifera* in Thailand but both *V. destructor* and *V. jacobsoni* were found on *A. cerana*. Again, none of the mite variants found on *A. cerana* was found on *A. mellifera* and vice versa.

The concatenated sequences of the K1-1 and J1-1 variants described are those of *V. destructor* collected from Taichung (Taiwan) and Tokyo (Japan), respectively (Tab. I), which had been previously identified using microsatellites as K1 and J1 types by Solignac et al. (2005).

GenBank accession numbers for the sequences of the 4 partial mitochondrial genes described here are given in Table I and their origin of detection in Asia is shown in Figure 1.

3.3. Nucleotide diversity

Standard sequence diversity indices (π) were computed from the mitochondrial concatenated sequences (2700 bp) for *V. destructor* collected on both *A. mellifera* and *A. cerana* (Tab. IV). The indice (π) for the 11 *V. destructor* haplotypes collected from *A. cerana* was $\pi = 0.0078$, whereas for the 7 *V. destructor* haplotypes carried by *A. mellifera* it was $\pi = 0.0047$, and for *V. destructor* isolates collected from both *A. cerana* and *A. mellifera* it was $\pi = 0.0075$. Sequences of only 2 individuals of *V. jacobsoni* were analyzed in this study and indices of diversity were not estimated. The two haplotypes (L1-1 and L1-2) differ by 2 point mutations in the *cox3* sequence.

3.4. Phylogenetic relationships

Sixty-six (66) nucleotides of the concatenated *V. destructor* sequences were polymorphic. An unrooted neighbour-joining tree of all *V. destructor* sequences showed two well-supported clades (Fig. 2), the first of which grouped variants of the Korea and Japan haplogroups and, the second, variants of the China

Table IV. Estimates of diversity and divergence of mitochondrial sequences of *Varroa* samples: (A) molecular diversity indices calculated for samples of *Varroa destructor* collected from *Apis cerana* and *Apis mellifera* colonies; (B) divergence between samples of *V. destructor* collected from *A. mellifera* and *A. cerana*.

Mite species	Bee host	Total sites	No. haplotypes (*A*)	No. segregating (*S*)	Nucleotide diversity (π)	Gene diversity (Hd)	Average No. nucleo differ (k)
(A) Molecular diversity							
Varroa destructor	*Apis cerana*	2700	11	62	0.00777	1 ± 0.034	20.97
Varroa destructor	*Apis mellifera*	2700	7	27	0.00466	0.917 ± 0.073	12.58
Varroa destructor	*A. cerana* & *A. mellifera*	1635	18	66	0.0075	0.986 ± 0.019	20.15
(B) Divergence between *V. destructor* on *A. mellifera* and *A. cerana*		2700		66	0.00746		20.15

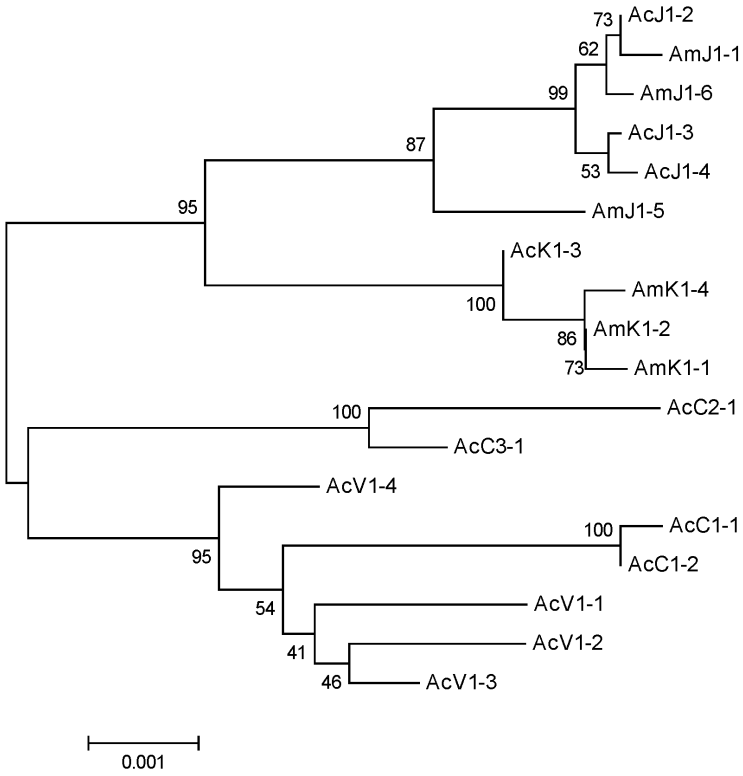

Figure 2. Phylogenetic relationships among haplotypes belonging to six different haplogroups (K1, J1, C1, C2, C3, V1) of *Varroa destructor* collected from two hosts, *Apis cerana (*Ac) and *A. mellifera (*Am) in Asia. Each haplotype is preceded by Am or Ac according to the host where the mite was sampled. Haplotypes were defined by sequences of 2700 nucleotides long of the mitochondrial genome spanning 4 genes (*cox1, cox3*, *atp6* and *cytb*).

and Vietnam haplogroups. A common lineage grouped all variants of the V1 haplotype together with the two variants of the C1 haplotype (95% bootstraps score), the two other China haplotypes (C2 and C3) remaining apart from this grouping.

4. DISCUSSION

Our study shows that the original K1 and J1 haplotypes of *V. destructor* on *A. mellifera* worldwide were derived from two distinct mite populations (represented by our K1 and J1 haplogroups, respectively) that infest *A. cerana* in north-east Asia. Both populations contain much more genetic variation than previously realised and newly discovered haplotypes in each population have successfully colonized *A. mellifera* in Asia, but have not yet spread from that region. These new haplotypes now represent new potential threats to *A. mellifera* outside of Asia.

4.1. Sensitivity of mitochondrial markers for detecting *Varroa* variation

Most variation detected to date among varroa isolates has been found using a single mtDNA marker. This marker, developed by Anderson and Fuchs (1998), was widely used by Anderson and Trueman (2000) to clarify the level of genetic variation among varroa mites on *A. cerana* throughout Asia and to help resolve varroa taxonomy. It has also been used in several more recent studies on varroa variation (Koeniger et al., 2002; Zhou et al., 2004;

Solignac et al., 2005; Warrit et al., 2006). The marker detects sequence variation in a 458 DNA base-pair region of the mt *cox1* gene. It can readily resolve an individual mite to the species level, as variation in this gene region is less than 2% among mites of the same species and more than 6% among mites of different species (Anderson and Trueman, 2000).

Here, we employed new longer mitochondrial sequences to determine how useful the *cox1* marker is for detecting varroa variation. It was not surprising that concatenated sequence data from the new sequences were far more sensitive at detecting variation than the *cox1* marker alone. However, it became obvious that, when used alone, the *cox1* marker was able to define particular populations of mites ('haplogroups'), whose members (haplotypes) were identified using the new markers. Hence, the combined use of the *cox1* marker and the new markers allows for detection of genetic variation both within and between different varroa populations. The sensitivity of the new mt sequences at detecting genetic variation falls between that of the less sensitive *cox1* marker and the more sensitive microsatellites used by Solignac et al. (2005).

4.2. Genetic variation among varroa mites on *A. cerana* in north-east Asia

We uncovered significant genetic diversity among varroa mites on *A. cerana* in north-east Asia (Tab. I; Tab. III). Although only 1 new haplogroup (C3 in Tab. I) was detected, 10 haplotypes were uncovered within haplogroups in which no genetic variation was previously known. Thus, we showed that varroa mites are more genetically variable on *A. cerana* in Asia than previously recognized.

Our results also showed that some closely-related varroa haplotypes on *A. cerana* had similar and quite restricted geographical distributions. For example, C1-1 and C1-2 in Figure 2 were both collected from the same province of Guangdong in China. On the other hand, members of some haplogroups such as those found in Thailand, China and Vietnam, were widely distributed. It is possible that the artificial movement of *A. cerana* in Asia by humans may have influenced such wide distributions. Hence, a better way of determining natural distributions may come from examining the associations of particular mite haplogroups with particular genotypes of *A. cerana*, as previous studies have noted that the biogeography of varroa haplotypes in Asia seems to correlate with the biogeography of *A. cerana* haplotypes (Anderson and Trueman, 2000; Warrit et al., 2006). Interestingly, none of the varroa haplotypes detected here on *A. cerana* was found on *A. mellifera* and vice-versa.

4.3. Genetic variation among varroa mites on *A. mellifera* in north-east Asia

As well as redefining the taxonomy of varroa, Anderson and Trueman (2000) also showed that K1 and J1 haplotypes of *V. destructor* were the only varroa mites to have successfully invaded and colonized *A. mellifera* globally. Solignac et al. (2005) verified those findings and further showed that there was almost no genetic variation between K1 and J1 (our K1-1 and J1-1) on *A. mellifera* for reasons that were not clear.

Here, *V. destructor* was the only varroa species detected on *A. mellifera* in north-east Asia and all haplotypes found were members of the K1 and J1 haplogroups. However, four (4) new haplotypes not previously reported on *A. mellifera* were also found (Tab. I; Tab. III). A new K1-2 haplotype was found on *A. mellifera* in Vietnam, a K1-2 and a K1-4 in China, a J1-5 in Thailand, and a J1-6 in Japan. The presence of these haplotypes in *A. mellifera* colonies at these locations is not unequivocal evidence that they have successfully colonized the colonies. Only mites that are able to reproduce on *A. mellifera* brood could successfully colonize and we do not have that information on the new haplotypes. The presence of varroa mites in an *A. mellifera* colony in Asia could simply indicate that the mites invaded the colonies from a local *A. cerana* colony without colonizing, as has been shown to occur for the V1-1 haplotype of *V. destructor* in Vietnam, a Java haplotype of *V. jacobsoni* in Indonesia

and for three unique varroa genotypes in the Philippines (Anderson, 1994; Fuchs et al., 2000). However, there is convincing evidence from our studies that at least 2 of the new haplotypes (J1-5 and K1-2) have successfully colonized *A. mellifera* in Asia. The most convincing evidence is the presence of J1-5 in *A. mellifera* colonies in Thailand. No member of the J1 haplogroup has ever been reported on *A. cerana* outside of Japan and certainly not in Thailand. J1-1 has been reported outside of Japan on *A. mellifera*, but it shows almost no genetic variation (Solignac et al., 2005). It has been known that a member of the J1 haplogroup has been present on *A. mellifera* in Thailand since 1997 (Anderson and Trueman, 2000). This same member was reported there again in 2001 (Warrit et al., 2006). Here we have shown that that member is J1-5, not J1-1. The only way J1-5 could have survived for 4 years in Thailand is if it could colonize *A. mellifera*. In our study we collected 10 mites from several different pupae in a single *A. mellifera* colony in Thailand and each was J1-5, which is way above the expected detection threshold if those mites were simply casual invaders of the colony (Anderson, 1994). Hence, it is clear that J1-5 has colonized *A. mellifera* in Asia. Our observations corroborate anecdotal evidence that *A. mellifera* was imported into Thailand from Japan via Taiwan as part of a bee extension program (Anderson, unpublished data).

The presence of K1-2 on *A. mellifera* in Vietnam and China provides less convincing evidence, but still substantial support, that haplotypes other than K1-1 and J1-1 have colonized *A. mellifera* in Asia. The detection of this haplotype in *A. mellifera* colonies was above the detection threshold expected for a casual invader and, interesting, like all other varroa haplotypes that have successfully invaded *A. mellifera*, we failed to detect K1-2 on *A. cerana*.

4.4. Insights into the geographical origins and invasive biology of *V. destructor*

Although there was no clear geographical structure of most mitochondrial haplotypes of *V. destructor* in Asia, most haplotypes of the J1 haplogroup (5 out of the 6) were found in Japan, confirming previous claims that this region was the origin of the J type (de Guzman et al., 1997; Anderson and Trueman, 2000). As for J1-1, evidence indicates it shifted to *A. mellifera* in Japan and was subsequently transported to Brazil during the 1970's (de Jong and Gonçalves, 1981; De Jong et al., 1982).

Whether or not all haplotypes of the K1 haplogroup colonizing *A. mellifera* originated from a discrete geographic region of Korea, as suggested by Warrit et al. (2006), needs further investigation. Here, we found K1-1 only on *A. mellifera* in Korea (Seoul) and Russia (Vladivostok), which lends support the hypothesis that K1-1 first colonized *A. mellifera* in Far-Eastern Russia (Danka et al., 1995) following a host-shift somewhere near Korea. Evidence suggests that the host-shift was made possible because mites of a single genetic linage (K1-1) were able to invade an *A. mellifera* colony from a colony of their indigenous *A. cerana* and were then able to lay eggs and produce offspring on the *A. mellifera* brood (Solignac et al., 2005). Our inability to find K1-1 on *A. cerana* in Asia lends further support to this hypothesis. After shifting to *A. mellifera*, K1-1 was then moved out of Asia into Europe, from where it spread globally.

By discovering new haplotypes of *V. destructor* colonizing *A. mellifera* in Asia, our studies suggest that the colonization of *A. mellifera* in Asia by new haplotypes is dependent on the length of exposure of those haplotypes to *A. mellifera*. Perhaps longer exposure eventually results in one type being selected for their ability to reproduce on *A. mellifera*. Given this scenario, we predict that other types of *V. destructor*, including those that are currently known to be non-reproductive on *A. mellifera*, will eventually colonize *A. mellifera* in Asia. Indeed, it is possible that such types have existed before in some parts of Asia, but may have completely exterminated *A. mellifera* and hence themselves.

In contrast to the *V. destructor* polymorphism unravelled in Asia, the *V. destructor* colonizing *A. mellifera* worldwide (K1-1 and J1-1) shows a complete lack of genetic diversity. These results are consistent with the

hypothesis of a bottleneck that occurred at the very beginning of the invasion of the Western honeybee by *V. destructor* as suggested by (Solignac et al., 2005). This scenario may have resulted from two independent events, one for each of the J and K types belonging to two distinct monophyletic haplogroups (Fig. 2). Due to varroa's rapid growth rate (about 12-fold increase in mite numbers yearly) (Martin, 1998), the situation is hardly compatible with the erosion of variability of *V. destructor* after colonizing *A. mellifera* colonies. A strong founder effect was also confirmed by the fact that J1-5 in Thailand, K1-2 in Vietnam and China, and J1-6 in Japan, which reproduce on *A. mellifera*, have been only found in Asia.

One of the main conclusions that have emerged from wider studies on invasive species is that the successful establishment of an exotic species into a new range is quite improbable and that many failed attempts occur before a successful invasion develops (Nivak, 2007). It has been often hypothesized that a selection of *ad hoc* genotypes operates at the beginning of an invasive process (Facon et al., 2006), and it can be speculated that the genotypes having invaded *A. mellifera* worldwide were pre-adapted to this new host. It is now known that some variation exists in the ability of varroa to reproduce on *A. cerana,* depending on the bee subspecies (Fuchs et al., 2000). Likewise, Warrit et al. (2006) suggest that populations of varroa on different host populations are genetically differentiated and may be adapted to specific characteristics of their local host bee populations. Understanding these adaptive characteristics may eventually lead to new ways of controlling invasive haplotypes on *A. mellifera*.

4.5. Implications for world apiculture

New *V. destructor* haplotypes that colonize *A. mellifera* may show slight differences in their life cycles, which cause them to become more virulent than the current K1-1 and J1-1. Indeed, J1-1 is assumed to be far less virulent to *A. mellifera* than K1-1 (de Guzman and Rinderer, 1999) and is thought to be displacing J1-1 in places where the two are sympatric (de Guzman et al., 1997; Anderson, 2000; Garrido et al., 2003). These observations caution against the free movement of honeybees and signal the need for strict and proper quarantine for the trade of live honeybees between countries.

ACKNOWLEDGEMENTS

We thank Prof. Z. Zeng and Prof. Wongsiri for providing sampling facilities in respectively China, and Thailand and S. Cros-Arteil and C. Moreau for help in sequencing. A part of the sequences in this work are from the graduate diploma, University Montpellier II, defended by J. Clement in 2004. Funding was provided by the European grant FEOGA (Fonds Europeen d'Orientation et de Garantie Agricole) ID B24000059 to MN and YLC, and a USDA-SCP grant to ZYH. Data used in this work were (partly) produced through molecular genetic analysis technical facilities of the IFR119 "Montpellier Environnement Biodiversité".

Nouveaux types asiatiques de *Varroa destructor* : une menace potentielle pour l'apiculture mondiale.

***Varroa destructor* / *Apis mellifera* / Asie / risque sanitaire / haplotype / ADN mitochondrial**

Zusammenfassung – Neue asiatische Typen von *Varroa destructor*: eine potentiell neue, weltweite Bedrohung für die Bienenhaltung. Die Varroamilbe, *Varroa destructor*, ist ein gut adaptierter Parasit der Östlichen Honigbiene (*Apis cerana*), insbesondere in den nördlichen Regionen des asiatischen Festlands. Mit ihrem Wechsel auf die Westliche Honigbiene (*A. mellifera*) im letzen Jahrhundert kam es zu einer dramatischen Ausbreitung dieser Milbe. Als verantwortlich für den Parasitenübergang werden zwei mitochondriale (mt) Haplotypen (K1 und J1) von *Varroa* angesehen. Bereits erhobene molekulare Daten deuten darauf hin, dass es sich hierbei, aufgrund der zeitlichen Zusammenhänge dieser Wirtswechsel, um zwei partiell isolierte Klone handelt. Mittels Genotypisierung von *V. destructor* Isolaten aus Regionen, in denen die Wirtswechsel der J1 und K1 Milben zuerst stattgefunden hatten, sowie einer weiträumigeren Untersuchung des natürlichen Ausbreitungsgebiets von *V. destructor* in Asien, versuchten wir in der vorliegenden Studie ein genaueres Bild über den Befall von *A. mellifera* durch *V. destructor* zu erhalten. Jede Milbenprobe wurde zunächst anhand des publizierten 458 Basenpaarfragments des

mitochondrialen Gens Cytochromoxidase 1 (*cox1*) genetisch charakterisiert, um neue Varianten bereits bekannten Haplotypen zuordnen zu können. Um die genetische Variabilität der Milben besser erfassen zu können, wurde im nächsten Schritt ein 2700 Nukleotide langes Fragment sequenziert, das vier mitochondriale Gene beinhaltet: *cox1*, Cytochromoxidase III (*cox3),* ATP Synthase 6 *(atp6)* und Cytochrom b (*cytb).* Wir untersuchten Varroamilben aus Asien, die sowohl die Östliche (21 Völker) als auch die Westliche Honigbiene (11 Völker) befallen hatten (Tab. I). Milben mit identischer Sequenz des 458 Basenpaare (bp) langen *cox1* Fragments wurden als Mitglieder jeweils einer der sieben Haplogruppen (K1, J1, V1, C1, C2, C3, L1) betrachtet. Insgesamt fanden wir 18 mt Haplotypen (Varianten der zusammenhängenden Sequenzen innerhalb einer Haplogruppe (Tab. III). Von diesen fanden wir 12 auf *A. cerana* und 6 auf *A. mellifera* (Tab. I, Abb. 1). Obwohl wir nur eine neue Haplogruppe fanden (C3 in Tab. I und III), konnten wir 12 neue Haplotypen innerhalb der Haplogruppen erkennen, für die bisher keine genetische Variation bekannt war. Dies zeigt, dass Varroamilben in Asien genetisch wesentlich variabler sind, als bisher angenommen. Unsere Untersuchung zeigt des weiteren, dass die weltweit auf *A. mellifera* gefundenden ursprünglichen K1 und J1 Haplotypen von *V. destructor* aus zwei unterschiedlichen Milbenpopulationen stammen. Diese sind jeweils durch die K1 und die J1 Haplogruppenzuordnung definiert (Abb. 2), die im nordöstlichen Asien *A. cerana* befallen. Beide Populationen sind genetisch wesentlich variabler, als bisher angenommen, und die hier neubeschriebenen Haplotypen dieser Populationen haben *A. mellifera* in Asien zwar befallen, sich aber noch außerhalb dieser Region verbreitet. Diese neuen Haplotypen stellen nun neue potentielle Bedrohungen für *A. mellifera* außerhalb Asiens dar. Außerdem stellen diese Beobachtungen eine Warnung gegen die freie Verfrachtung von Honigbienen dar und zeigen die Notwendigkeit einer strengen und korrekten Quarantäne für den internationalen Handel mit lebenden Honigbienen.

***Varroa* / *Apis mellifera* / *Apis cerana* / mitochondriale DNA / Diversität**

REFERENCES

Anderson D.L. (1994) Non-reproduction of *Varroa jacobsoni* in *Apis mellifera* colonies in Papua New Guinea and Indonesia, Apidologie 25, 412–421.

Anderson D.L. (2000) Variation in the parasitic bee mite *Varroa jacobsoni* Oud., Apidologie 31, 281–292.

Anderson D.L., Fuchs S. (1998) Two genetically distinct populations of *Varroa jacobsoni* with contrasting reproductive abilities on *Apis mellifera*, J. Apicult. Res. 37, 69–78.

Anderson D.L., Trueman J.W.H. (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species, Exp. Appl. Acarol. 24, 165–189.

Clement M., Posada D., Crandall K.A. (2000) TCS: a computer program to estimate gene genealogies, Mol. Ecol. 9, 1657–1659.

Crane E. (1978) The Varroa mite, Bee World 59, 164–167.

Danka R., Rinderer T.E., Kuznetsov V., Delatte G. (1995) A USDA-ARS project to evaluate resistance to *Varroa jacobsoni* by honey bees of Far-Eastern Russia, Am. Bee J. 135, 746–748.

de Guzman L.I., Rinderer T.E. (1999) Identification and comparison of *Varroa* species infesting honey bees, Apidologie 30, 85–95.

de Guzman L.I., Rinderer T.E., Stelzer J.A. (1997) DNA evidence of the origin of *Varroa jacobsoni* Oudemans in the Americas, Biochem. Genet. 35, 327–335.

de Guzman L.I., Rinderer T.E., Stelzer J.A. (1999) Occurrence of two genotypes of *Varroa jacobsoni* Oud. in North America, Apidologie 30, 31–36.

de Jong D., Gonçalves L.S. (1981) The Varroa problem in Brazil, Am. Bee J. 121, 186–189.

de Jong D., Morse R.A., Eickwort G.C. (1982) Mites pests of honey bees, Annu. Rev. Entomol. 27, 229–252.

Evans J., Lopez D.L. (2002) Complete Mitochondrial DNA Sequence of the Important Honey Bee Pest, *Varroa destructor* (Acari: Varroidae), Exp. Appl. Acarol. 27, 69–78.

Facon B., Genton B.J., Shykoff J., Jarne P., Estoup A., David P. (2006) A general eco-evolutionary framework for understanding bioinvasions, Trends Ecol. Evol. 21, 130–135.

Fuchs S., Long L., Anderson D. (2000) A scientific note on the genetic distinctness of *Varroa* mites on *Apis mellifera* L. and on *Apis cerana* Fabr. in North Vietnam, Apidologie 31, 456–460.

Garrido C., Rosenkranz P., Paxton R.J., Gonçalves L. (2003) Temporal changes in *Varroa destructor* fertility and haplotype in Brazil, Apidologie 34, 535–541.

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic Acids Symp. 41, 95–98.

Koeniger G., Koeniger N., Anderson D.L., Lekprayoon C., Tingek S. (2002) Mites from debris and sealed brood cells of *Apis dorsata* colonies in Sabah (Borneo) Malaysia, including a new haplotype of *Varroa jacobsoni*, Apidologie 33, 15–24.

Kumar S., Tamora K., Nei M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment, in: Briefings in Bioinformatics, The Pennsylvania

State University, University Park, PA 16802, pp. 150–163.

Martin S. (1998) A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies, Ecol. Model. 109, 267–281.

Navajas M., Solignac M., Le Conte Y., Cros-Arteil S., Cornuet J.M. (2002) The complete sequence of the mitochondrial genome of the honey-bee ectoparasite *Varroa destructor* (Acari:Mesostigmata), Mol. Biol. Evol. 19, 2313–2317.

Nei M. (1987) Molecular evolutionary genetics, First ed., Columbia University Press, New York.

Nivak S.J. (2007) The role of evolution in the invasion process, Proc. Natl Acad. Sci. (USA) 104, 3671–3672.

Oldroyd B.P. (1999) Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees, Trends Ecol. Evol. 8, 312–315.

Oudemans A. (1904) Nog iets aangaande de "Afbeeldingen met beschrijving van insecten, schdelijk voor naaldhout", Berichten 18, 156–164.

Rozas J., Sanchez-DelBarrio J.C., Messeguer X., Rozas R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods, Bioinformatic 29, 2496–2497.

Sakai T., Okada I. (1973) The present beekeeping in Japan, Glean Bee Cult. 101, 356–357.

Sammataro D., Gerson U., Needham G. (2000) Parasitic mites of honey bees: life history, implications and impact, Annu. Rev. Entomol., 519–548.

Solignac M., Cornuet J., Vautrin D., Le Conte Y., Anderson D., Evans J., Cros-Arteil S., Navajas M. (2005) The invasive Russian and Japanese types of *Varroa destructor*, ectoparasite mite of the Western honey bee (*Apis mellifera*), are two partially isolated clones, Proc. R. Soc. London B 272, 411–419.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through weighting, position-specific gap penalties and weight matrix, Nucleic Acids Res. 22, 4673–4680.

Warrit N., Smith D.R., Lekprayoon C. (2006) Genetic subpopulations of *Varroa* mites and their *Apis cerana* hosts in Thailand, Apidologie 37, 19–30.

Zhou T., Anderson D., Huang Z.S.H., Yao J., Tan K., Zhang Q. (2004) Identification of Varroa mites (Acari: Varroidae) infesting *Apis cerana* and *Apis mellifera* in China, Apidologie 35, 645–654.