Apidologie (2011) 42:485–498

DOI: 10.1007/s13592-011-0028-6

Original article

Identification of *Tropilaelaps* mites (Acari, Laelapidae) infesting *Apis mellifera* in China

Qi Hua Luo[1,2], Ting Zhou[1,2], Qiang Wang[1,2], Ping Li Dai[1,2], Yan Yan Wu[1,2], and Huai Lei Song[1,2]

[1]Institute of Apicultural Research, Chinese Academy of Agricultural Science, Xiangshan Road, Beijing 100093, People's Republic of China
[2]Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Beijing 100093, China

Received 28 December 2009 – Revised 1 August 2010 – Accepted 17 August 2010

Abstract – *Tropilaelaps* mite samples were collected from 72 locations in 25 provinces throughout China. The mitochondrial DNA *cox*1 and ribosomal ITS1-5.8S-ITS2 fragments of *Tropilaelaps* mites were surveyed for sequence variation or the presence or absence of specific restriction sites that differentiate four species of *Tropilaelaps* mite (*Tropilaelaps clareae*, *Tropilaelaps mercedesae*, *Tropilaelaps koenigerum*, and *Tropilaelaps thaii*). Based on these identified diagnostic characters, all samples in this study corresponded to *T. mercedesae*, which has been mistaken for *T. clareae* until now. None of the other *Tropilaelaps* species were found infesting *Apis mellifera* in China. A total of 73 *cox1* haplotypes and 104 ITS1-5.8S-ITS2 haplotypes were discovered in this study. Haplotype analysis revealed that there is no association between geographic distance and genetic distance among populations. The results of the present study clarified the taxonomic status and biogeography of *Tropilaelaps* mites in China, and should have implications for the control and bee quarantine efforts in China.

Tropilaelaps mercedesae **/ identification /** ***Apis mellifera*** **/ mtDNA /** ***cox1*** **/ ITS1-5.8S-ITS2 / China**

1. INTRODUCTION

Mites of the genus *Tropilaelaps* are ectoparasites of honeybees *Apis* native to Asia (Delfinado and Baker 1961; Laigo and Morse 1968). The primary host of *Tropilaelaps* mites was recognized as the "giant" honeybee, *Apis dorsata* (Laigo and Morse 1968; Anderson and Morgan 2007), but they are able to infest a wide spectrum of honeybee species raging from *Apis mellifera*, *Apis cerana*, *Apis dorsata*, *Apis florae* and *Apis laboriosa* (Bailey and Ball 1991; Schmid-Hempel 1998). However, *Tropilaelaps* mites appear to be particularly problematic in *A. mellifera* (Burgett et al. 1983; de Jong et al. 1982; Laigo and Morse 1969). Similar to *Varroa destructor* Anderson and Trueman (Acari: Varroaidae), *Tropilaelaps* mites infect brood, suck haemolymoph, and can cause brood malformation, death of individual bees, and subsequent colony decline or absconding.

At least four species of *Tropilaelaps* have been recognized, namely *Tropilaelaps clareae* Delfinado and Baker, *Tropilaelaps mercedesae* Anderson and Morgan, *Tropilaelaps koenigerum* Delfinado-Baker and Baker and *Tropilaelaps thaii* Anderson and Morgan. *T. clareae* is a parasite of the native honey bee *Apis dorsata breviligula* and is also a parasite of the introduced honey bee *A. mellifera* in the Philippines and the native honey bee species *Apis dorsata binghami* on the Sulawesi Island in Indonesia. *T. mercedesae*, which has been mistaken as *T. clareae* until now, together with *T. koenigerum*, parasitizes the native *Apis dorsata dorsata* in mainland Asia and Indonesia (except the Sulawesi Island). *T. mercedesae* is

Corresponding author: T. Zhou, ztapis@263.net
Manuscript editor: Marina Meixner

also a parasite of *A. mellifera* in these and surrounding regions and, together with another species, *T. thaii*, also parasitises *A. laboriosa* in mountainous Himalayan regions (Anderson and Morgan 2007).

In China, *Tropilaelaps* mites are serious ectoparasites of *A. mellifera* (Zeng 2003; Luo et al. 2008), but the true identity of *Tropilaelaps* mites infesting *A. mellifera* has been poorly studied. Initially, the mites detected on *A. mellifera* in China have been thought to be *T. clareae* (Woyke 1994). But recently two samples from *A. mellifera* and one sample from *A. dorsata dorsata* in China were identified as *T. mercedesae* by Anderson and Morgan (2007). This study suggested that the *T. mercedesae* has been observed in *A. mellifera* and *A. dorsata* in China, but it is still unclear how many species of *Tropilaelaps* mites occur in China and whether *A. mellifera* is parasitized by more than one species of *Tropilaelaps* mites.

Morphological studies of *Tropilaelaps* species were reported by Anderson and Morgan (2007), while differentiation of *Tropilaelaps* mites based principally on morphology is difficult and requires taxonomical skills. More recently, nuclear ribosomal DNA (rDNA) has been widely used to differentiate closely related organisms, particularly at interspecific level (Hillis and Davis 1986; Hillis and Dixon 1991; Odorico and Miller 1997). Mitochondrial DNA (mtDNA) has also been widely employed in phylogenetic studies of animals, because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species (Brown et al. 1979; Moore 1995; Mindell et al. 1997).

In this study, we surveyed samples of *Tropilaelaps* mites for sequence variation and diagnostic restriction sites in the mtDNA *cox1* and nuclear rDNA ITS1-5.8S-ITS2 regions, because a previous study (Anderson and Morgan 2007) has shown that these genetic markers were useful in distinguishing four species of *Tropilaelaps* mites: *T. clareae*, *T. mercedesae*, *T. koenigerum*, and *T. thaii*. We also analyzed the genetic variation and geographic distribution of *cox1* and ITS1-5.8S-ITS2 haplotypes.

2. MATERIALS AND METHODS

2.1 Sampling and DNA extraction

Samples of adult *Tropilaelaps* mites were collected from 72 different locations in 25 provinces throughout China in 2007, 2008, and 2009. The locations and collection details of the *Tropilaelaps* mites examined are described in Table I and shown in Figure 1, the geographic coordinates for the sampling locations are provided in Table II. Mites were collected alive from the capped worker or drone brood cells, and some mites were also collected from the hive bottom boards within 24 h following the placement of plastic strips impregnated with acaricide (Apistan) between brood frames. All mites were killed and preliminarily identified based on morphology according to Anderson and Morgan (2007). Samples were preserved in absolute ethanol and kept at −20°C until laboratory processing.

Total DNA was extracted from each mite following the procedure of Garnery et al. (1993). A total of 210 mites were subjected to both *cox1* and ITS1-5.8S-ITS2 sequence analyses, and additional 429 mites were used for analysis by polymerase chain reaction (PCR)–restriction fragment length polymorphism analysis.

2.2 PCR amplification and sequencing of *cox1* and ITS1-5.8S-ITS2

The *cox1* region of the mtDNA was amplified by PCR (Saiki 1990) using the following thermal profile: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 30 s, and a final extension of 10 min at 72°C. The ITS1-5.8S-ITS2 region was amplified following the same PCR conditions with the exception of annealing temperature which was 54°C. The reaction volume of 50 μL contained 50 ng genomic DNA, 1.0 μmol/L of each primer, 500 μmol/L of each dATP, dGTP, dTTP, and dCTP, and 1.5 units of Takara Taq polymerase. Reactions were buffered by addition of the PCR buffer (Takara Shuzo Co., Ltd.) containing 1.75 mmol/L $MgCl_2$. The primers for amplifying *cox1* and ITS1-5.8S-ITS2 were described by Anderson et al. (1998) and White et al. (1990) respectively.

The PCR products were purified and cloned into the T-Easy vector (Promega). The recombinant plasmid was used to transform the competent

Table I. Details of the *Tropilaelaps* samples examined and their identity (genotype), based on mitochondrial *cox1* and ITS1-5.8S-ITS2 sequences analyses

Site[a]	Province (district or locality)	No. of colonies/apiaries sampled	Sample abbreviation	Number of mites: IS/SS/IE/SE	Identity of mites: Species (haplotype Cn; Sn)
1	Hainan (Danzhou)	9/2	HN1	6/6/8/8	Tm (C1, C2, C3, C4, C5; S6)
2	Hainan (haikou)	13/2	HN2	5/5/8/8	Tm (C1, C2, C3, C4; S7)
3	Yunnan (chuxiong)	3/1	YN1	2/2/4/4	Tm (C3; S29)
4	Yunnan (Kunming)	4/1	YN2	3/3/3/3	Tm (C3, C65, C70; S73)
5	Guangxi (Liuzhou)	6/2	GX1	3/3/8/8	Tm (C12, C15; S6, S13)
6	Guangdong (Guangzhou)	5/1	GD1	2/2/3/3	Tm (C3, C28; S14)
7	Guangdong (Dongguan)	9/2	GD2	3/3/4/4	Tm (C3, C14; S15, S29)
8	Guangdong (Heyuan)	2/1	GD3	3/3/1/1	Tm (C3, C28; S13, S31, S96)
9	Sichuan (Panzhihua)	16/1	SC4	4/4/4/4	Tm (C3, C11, C22; S14, S18, S29, S31)
10	Guizhou (Liupanshui)	3/1	GZ1	2/2/3/3	Tm (C3, C29; S13, S18)
11	Guizhou (Anshun)	4/1	GZ2	2/2/4/4	Tm (C3; S78)
12	Guizhou (Zhengan)	4/1	GZ3	9/9/4/4	Tm (C3, C56, C57, C58; S13, S18, S74, S75, S76, S77, S82, S89, S94)
13	Guizhou (Meitan)	23/3	GZ4	6/6/8/8	Tm (C3; S13, S29, S80, S90, S91, S102)
14	Guizhou (Tongren)	3/1	GZ5	2/2/3/3	Tm (C3, C68; S58, S79,)
15	Guizhou (Wuchuan)	4/1	GZ6	4/4/3/3	Tm (C3, C71; S13, S81, S92, S93)
16	Hunan (Shaoyang)	3/1	HuN1	2/2/2/2	Tm (C3; S3, S13)
17	Hunan (Hengyang)	8/1	HuN2	2/2/4/4	Tm (C3; S13)
18	Hunan (Loudi)	3/1	HuN3	2/2/2/2	Tm (C66; S13, S18)
19	Hunan (Changsha)	3/2	HuN4	3/3/4/4	Tm (C3, C67; S29)
20	Hunan (Miluo)	3/1	HuN5	2/2/2/2	Tm (C66; S87, S101)
21	Jiangxi (Yihuang)	4/1	JX1	2/2/8/8	Tm (C3, C19; S13, S19)
22	Jiangxi (Fuzhou)	4/1	JX2	2/2/7/7	Tm (C3; S21, S23)
23	Jiangxi (Ruichang)	5/2	JX3	3/3/8/8	Tm (C3, C21; S29, S40, S41)
24	Jiangxi (Wannian)	3/2	JX4	4/4/9/9	Tm (C3, C39; S18, S18, S20, S71)
25	Fujian (Sanming)	6/1	FJ1	2/2/8/8	Tm (C3; S3, S29)
26	Fujian (Quanzhou)	4/2	FJ2	1/1/6/6	Tm (C3, C46; S13)

Table I (continued)

Site[a]	Province (district or locality)	No. of colonies/apiaries sampled	Sample abbreviation	Number of mites: IS/SS/IE/SE	Identity of mites: Species (haplotype Cn; Sn)
27	Fujian (Fuzhou)	4/1	FJ3	2/2/6/6	Tm (C54; S13)
28	Zhejiang (Jinhua)	2/1	ZJ1	2/2/2/2	Tm (C3, C30; S13, S17)
29	Zhejiang (Hangzhou)	5/1	ZJ2	2/2/6/6	Tm (C3, C63; S80, S100)
30	Sichuan (Yajiang)	13/2	SC1	5/5/9/9	Tm (C3, C40, C43; S54, S66, S68)
31	Sichuan (Santai)	4/1	SC2	2/2/5/5	Tm (C3, C38; S13, S67)
32	Sichuan (Pengxi)	14/2	SC3	2/2/3/3	Tm (C3, C44; S15, S64)
33	Chongqing (Rongchang)	7/1	CQ1	4/4/7/7	Tm (C3, C31, S13, S18, S43, S44)
34	Chongqing (Nanchuan)	26/3	CQ2	6/6/7/7	Tm (C3, C59; S83, S84, S85, S86)
35	Hubei (Jingzhou)	4/1	HuB1	2/2/6/6	Tm (C3, C17; S8, S9)
36	Hubei (Suizhou)	34/1	HuB2	4/8/8/8	Tm (C3, C20, C23, C40; S13, S18, S15, S24, S26, S29, S30, S54,)
37	Hubei (Guangshui)	12/1	HuB3	2/2/8/8	Tm (C3, C47; S13, S55)
38	Hubei (Ezhou)	16/3	HuB4	6/6/9/9	Tm (C3, C64, C69; S13, S28, S56, S59, S60, S73)
39	Anhui (Huangshan)	5/2	AH1	3/3/7/7	Tm (C3, C8; S15, S32)
40	Anhui (Chizhou)	23/2	AH2	4/4/5/5	Tm (C9, C10, C11; S1, S11)
41	Anhui (Hefei)	4/1	AH3	2/2/7/7	Tm (C3, C55; S61, S62)
42	Jiangsu (Nanjing)	4/1	JS1	2/2/4/4	Tm (C3; S18, S36)
43	Jiangsu (Wuxi)	9/2	JS2	4/4/5/5	Tm (C33, C51; S13, S37, S43)
44	Jiangsu (Haian)	2/2	JS3	2/2/6/6	Tm (C3, C52; S29)
45	Gansu (Longnan)	11/1	GS1	2/2/4/4	Tm (C3; S15)
46	Gansu (Tianshui)	4/2	GS2	2/2/7/7	Tm (C3; S15, S58)
47	Ningxia (Guyuan)	2/2	NX1	4/4/7/7	Tm (C3, C62, C41; S37, S97, S98, S99)
48	Shanxi (Fengxiang)	3/1	SXw1	2/2/6/6	Tm (C3, C25; S15, S18)
49	Shanxi (Hanzhong)	7/1	SXw2	2/2/8/8	Tm (C3, C34; S13, S52)
50	Shanxi (Sanyuan)	4/2	SXw3	2/2/7/7	Tm (C3, C35; S46, S63)
51	Henan (Nanyang)	7/2	HeN1	4/4/7/7	Tm (C20, C24; S22, S27, S18)
52	Henan (Luoyang)	4/1	HeN2	2/3/5/5	Tm (C3, C26; S13, S25, S15)

Table I (continued)

Site[a]	Province (district or locality)	No. of colonies/apiaries sampled	Sample abbreviation	Number of mites: IS/SS/IE/SE	Identity of mites: Species (haplotype Cn; Sn)
53	Henan (Kaifeng)	5/2	HeN3	2/2/7/7	Tm (C3, C27; S18, S50)
54	Henan (Anyang)	3/1	HeN4	2/2/5/5	Tm (C3; S15)
55	Shandong (Jinan)	15/2	SD1	4/4/8/8	Tm (C3, C13, C18; S13, S15, S45, S53)
56	Shandong (Zibo)	14/2	SD2	2/2/11/11	Tm (C3, C32; S16,S17)
57	Shandong (Dezhou)	3/1	SD3	2/3/8/8	Tm (C32, C42; S3, S38, S39)
58	Shanxi (Yunchen)	4/1	SXe1	2/2/6/6	Tm (C3; S3, S13)
59	Shanxi (Changzhi)	3/2	SXe2	3/3/12/12	Tm (C30, C37; S20, S33, S47)
60	Shanxi (Yangquan)	4/2	SXe3	2/4/9/9	Tm (C3, C37; S15, S65, S37, S51)
61	Hebei (Tanshan)	5/2	HeB1	2/2/8/8	Tm (C3; S29)
62	Hebei (Shunping)	6/1	HeB2	2/2/5/5	Tm (C29; S42)
63	Hebei (Guan)	5/1	HeB3	2/2/6/6	Tm (C25; S34, S35)
64	Hebei (Tangxian)	4/2	HeB4	2/2/10/10	Tm (C3, C49; S4, S5)
65	Hebei (Longhua)	5/1	HeB5	2/2/6/6	Tm (C3, C50; S15, S58)
66	Beijing (Fangshan)	34/4	BJ1	2/2/6/6	Tm (C16; S13, S17)
67	Beijing (Haidian)	12/3	BJ2	3/3/6/6	Tm (C45; S2, S3)
68	Liaoning (Xinchen)	10/3	LN1	3/3/12/12	Tm (C3, C48; S18, S57, S69)
69	Liaoning (Tieling)	25/1	LN2	2/2/5/5	Tm (C3; S70, S72)
70	Neimeng (Chifeng)	7/2	NM1	2/2/6/6	Tm (C3, C53; S15, S49)
71	Chongqing (Jiangjing)	3/1	CQ3	2/3/2/2	Tm (C60, C61; S48, S13, S95)
72	Chongqing (Dadukou)	3/1	CQ4	2/2/2/2	Tm (C72, C73; S13, S104)

IS cox1 sequences analyses, *SS* ITS1-5.8S-ITS2 sequence analyses, *IE* restriction analyses of *cox*1 regions; SE = restriction analyses of ITS1-5.8S-ITS2.regions, *Tm* T. mercedesae, *C* haplotype based on *cox1* sequence, *S* haplotype based on ITS1-5.8S-ITS2 sequences, *n* haplotype numbers

[a] Sites correspond with locations in Figure 1

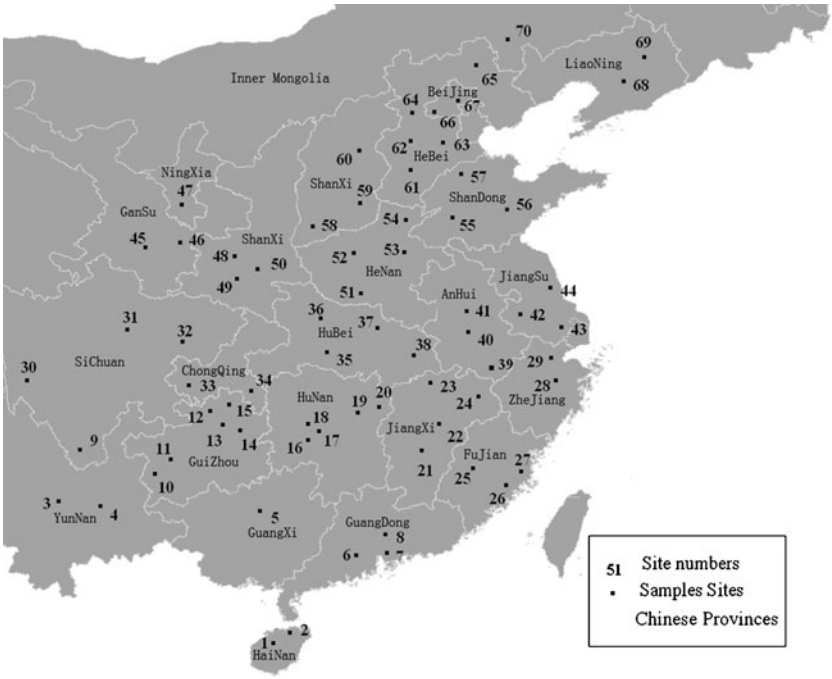

Figure 1. Map of China showing sampling locations (*1–70*) of mite specimens used in this study. Names of the localities are given in Table I.

Escherichia coli DH-5a cells. Positive clones were screened and subjected to sequencing using ABI-377 (Applied Biosystems)

2.3 Restriction analysis of amplified *cox1* and ITS

Species-specific markers were used as described by Anderson and Morgan (2007) to confirm the identity of *Tropilaelaps* mites of China. *Cox1* fragments were digested with restriction enzymes *Fau*I, *Bsr*I, *Bst*YI, *Swa*I, and ITS1-5.8S-ITS2 with *Rsa*I. The digests were analyzed by electrophoresis through 2.0% MetaPhor agarose gels (FMC) and visualized under a UV transilluminator after staining with ethidium bromide.

2.4 Data analysis

The nucleotide sequences were analyzed using BLAST (Altschul et al. 1990) and Clustal W (Thompson et al. 1994). The genetic diversity of the haplotypes (*H*) and nucleotides (*P*) was calculated by using the DnaSP program (Rozas et al. 2003). Phylogenetic relationships among haplotypes were estimated by using the MEGA program (Kumar et al. 2004), based on the neighbor-joining (NJ) algorithm and the Tamura–Nei genetic distance model. Bootstrap support was calculated by means of 1,000 replicates, with 70% support considered well-supported (Hillis and Bull 1993; Efron et al. 1996). In order to confirm the identity of *Tropilaelaps* mites in China, the mtDNA *cox1* haplotypes in this study were compared with those available in previous inquiries, from Thailand, EF025445.1 (*T. mercedesae*); Vietnam, EF025452.1 (*T. thaii*); Mindoro, EF025464.1 (*T. clareae*); Sumatra, EF025451.1 (*T. koenigerum*); and the ITS1-5.8S-ITS2 haplotypes were compared with those from Sri Lanka, EF025472.1 (*T. mercedesae*); Thailand, AF544013.1 (*T. clareae*); Vietnam, EF025477.1 (*T. thaii*);

Table II. Geographic coordinates for sampling locations of mite specimens used in this study

Site[a]	Province	District or locality	Longitude	Latitude
1	Hainan	Danzhou	109.58313 E	19.526142 N
2	Hainan	Haikou	110.360413 E	20.024968 N
3	Yuannan	Chuxiong	101.539764 E	25.055745 N
4	Yuannan	Kunming	102.757874 E	25.073783 N
5	Guangxi	Liuzhou	109.416275 E	24.317691 N
6	Guangdong	Guangzhou	113.26355 E	23.216107 N
7	Guangdong	Dongguan	113.774414 E	23.074678 N
8	Guangdong	Heyuan	114.686279 E	23.750154 N
9	Sichuan	Panzhihua	101.724472 E	26.581159 N
10	Guizhou	Liupanshui	104.82811 E	26.594053 N
11	Guizhou	Anshun	105.941849 E	26.256781 N
12	Guizhou	Zhengan	107.438049 E	28.557988 N
13	Guizhou	Meitan	107.471008 E	27.749177 N
14	Guizhou	Tongren	109.186249 E	27.720005 N
15	Guizhou	Wuchuan	107.892609 E	28.533862 N
16	Hunan	Shaoyang	111.460419 E	27.318705 N
17	Hunan	Hengyang	112.372284 E	26.96859 N
18	Hunan	Loudi	111.994972 E	27.699944 N
19	Hunan	Changsha	113.080902 E	28.253586 N
20	Hunan	Miluo	113.080902 E	28.881957 N
21	Jiangxi	Yihuang	116.216125 E	27.522887 N
22	Jiangxi	Fuzhou	116.39019 E	27.998342 N
23	Jiangxi	Ruichang	115.666466 E	29.673735 N
24	Jiangxi	Wannian	117.068939 E	28.694804 N
25	Fujian	Sanming	117.641258 E	26.264478 N
26	Fujian	Quanzhou	118.590546 E	24.906367 N
27	Fujian	Fuzhou	119.261055 E	26.079913 N
28	Zhejiang	Jinhua	119.609871 E	29.100877 N
29	Zhejiang	Hangzhou	120.070953 E	30.420256 N
30	Sichuan	Yajiang	101.008301 E	29.993002 N
31	Sichuan	Santai	105.095215 E	31.088222 N
32	Sichuan	Pengxi	105.718689 E	30.772519 N
33	Chongqing	Rongchang	105.593033 E	29.391748 N
34	Chongqing	Nanchuan	107.09198 E	29.152161 N
35	Hubei	Jingzhou	112.17762 E	30.369247 N
36	Hubei	Suizhou	113.384743 E	31.658934 N
37	Hubei	Guangshui	113.779221 E	31.545186 N
38	Hubei	Ezhou	114.88678 E	30.409005 N
39	Anhui	Huangshan	118.405838 E	29.722048 N
40	Anhui	Chizhou	117.499294 E	30.681916 N
41	Anhui	Hefei	117.310638 E	31.87989 N
42	Jiangsu	Nanjing	119.083557 E	32.056973 N

Table II (continued)

Site[a]	Province	District or locality	Longitude	Latitude
43	Jiangsu	Wuxi	120.274887 E	31.589064 N
44	Jiangsu	Haian	120.480194 E	32.552022 N
45	Gansu	Longnan	104.932995 E	33.406081 N
46	Gansu	Tianshui	105.713882 E	34.575278 N
47	Ningxia	Guyuan	106.2323 E	36.02439 N
48	Shanxi	Fengxiang	107.378311 E	34.532581 N
49	Shanxi	Hanzhong	107.058334 E	33.076008 N
50	Shanxi	Sanyuan	108.927383 E	34.637163 N
51	Henan	Nanyang	112.508926 E	33.003481 N
52	Henan	Luoyang	112.48558 E	34.627558 N
53	Henan	Kaifeng	114.321671 E	34.760794 N
54	Henan	Anyang	114.349823 E	36.132329 N
55	Shandong	Jinan	117.000275 E	36.689896 N
56	Shandong	Zibo	117.92038 E	36.836218 N
57	Shandong	Dezhou	116.325989 E	37.507002 N
58	Shanxi	Yunchen	110.965004 E	35.001878 N
59	Shanxi	Changzhi	113.15609 E	36.195525 N
60	Shanxi	Yangquan	113.571854 E	37.884067 N
61	Hebei	Tanshan	118.181305 E	39.632134 N
62	Hebei	Shunping	115.138092 E	38.878205 N
63	Hebei	Guan	116.315002 E	39.452631 N
64	Hebei	Tangxian	114.98291 E	38.743373 N
65	Hebei	Longhua	117.729492 E	41.304634 N
66	Beijing	Fangshan	116.146088 E	39.751017 N
67	Beijing	Haidian	116.201706 E	40.012628 N
68	Liaoning	Xinchen	120.727386 E	40.614995 N
69	Liaoning	Tieling	123.812828 E	42.310577 N
70	Neimeng	Chifeng	118.868637 E	42.281119 N
71	Chongqing	Jiangjing	106.260452 E	29.291789 N
72	Chongqing	Dadukou	106.482925 E	29.486528 N

Lombok-EF025475.1 (*T. koenigerum*). All of these sequences except AF544013.1 (Tangjingjai et al. 2003) were from Anderson and Morgan (2007).

3. RESULTS

All mites in this study were preliminarily identified as *T. mercedesae* based on morphology according to Anderson and Morgan (2007). The identities of mites were confirmed by their *cox1* and ITS sequences or restriction enzyme analyses are summarized in Table I.

3.1 Analysis of *cox1* sequences

All of the amplified *cox1* fragments from *Tropilaelaps* mite in this study were 538 bp in length, and showed a high level of similarity (97–100%) to that of *T. mercedesae* described by Anderson and Morgan (2007), but lower similarity to *T. thaii* (88%), *T. clareae* (88%), *T.*

koenigerum (86%). There was a difference between all *Tropilaelaps* mite in the present study and previously reported *T. mercedesae* at the site of 514 bp (Figure 2), they were G:C base pairs instead of T:A base pairs. Alignment of the *cox1* sequences of *Tropilaelaps* mite in China were shown in Figure 2 [the GenBank accession no. were HQ533148 (HN5), HQ533149 (JX3), HQ533150(HeB3), HQ533151(SXe2), HQ533152(GD1), HQ533153(SXw2), HQ533154(YN2), HQ533155(GZ6), HQ533156 (HuB2), HQ533157(GX1), HQ533158(NX1), and HQ533159(LN1)]. *Cox1* sequences of *T. mercedesae* in this study exhibited low levels of nucleotide diversity (*P*): 0.00396±0.00018 (mean±standard), while, haplotypic diversity (*H*) was relatively high: 0.654±0.044. A total of 73 haplotypes were found (sites with alignment gaps were considered) from 202 sequenced individuals (eight mites were not successfully amplified), the haplotype C3 being the most frequent (53.96%). Haplotype analysis revealed that there was no association between geographic distance and genetic distance among populations, because identical haplotypes have been widely distributed geographically (e.g., C3).

Phylogenetic analyses performed under all three optimality criteria (maximum parsimony, maximum likelihood, and neighbor-joining) produced similar results. Hence, only the NJ analyses are reported here. Branches supported by more than 50% of the bootstrap replicates were indicated (Figure 3). NJ analysis revealed that all haplotypes in China grouped closely with haplotype from *T. mercedesae* recently described by Anderson and Morgan (2007). Indeed, all of the *cox1* haplotypes in China and *T. mercedesae* (data available in GenBank) formed tight, monophyletic clusters supported by high bootstrap values (99%). The NJ topology also supported that these mites were separable from the *T. clareae*, *T. koenigerum*, and *T. thaii* described by Anderson and Morgan (2007).

3.2 ITS1-5.8S-ITS2 sequences analysis

The ITS4 and ITS5 primers amplified a single DNA fragment from each of the mite samples. The sequences were 99% similar to the ITS1-5.8S-ITS2 sequence of *T. mercedesae*, 96% similar to *T. clareae*, 96% similar to *T. thaii* and 92% similar to *T. koenigerum* described by Anderson and Morgan (2007). Alignment of the ITS1-5.8S-ITS2 sequences of *Tropilaelaps* mite in China is shown in Figure 4 [the GenBank accession no. were HQ533160 (CQ1), HQ533161(HuB1), HQ533162(HN1), HQ533163(GZ3), HQ533164(HuN1), HQ533165(SXe3), HQ533166(SXw1), HQ533167(NM1), HQ533168(ZJ2), HQ533169 (CQ2), HQ533170(HuN1), and HQ533171 (AH2)]. ITS1-5.8S-ITS2 sequences of *T. mercedesae* in this study exhibited low levels of nucleotide diversity (*P*): 0.00479±0.00057, while haplotypic diversity (*H*) was relatively high: 0.8691±0.020. A total of 104 haplotypes (sites with alignment gaps were considered) were discovered from 210 sequenced individuals (Table I). Haplotype S13, S15, and S18 were the most frequent (10.47%, 10%, and 8.57% respectively). Haplotype analysis also revealed that there was no association between geographic distance and genetic distance among populations.

Phylogenetic analysis of the ITS haplotypes resulted in a neighbor-joining tree (Figure 5) that was similar to that based on mitochondrial data (Figure 3). Branches that were supported by more than 50% of the neighbor-joining bootstrap replicates are indicated. Phylogenetic relationship analysis also revealed that all ITS1-5.8S-ITS2 haplotypes in this study grouped in one cluster with *T. mercedesae* described by Anderson and Morgan (2007), even though the bootstrap value was only moderately high (72%). This tree also indicated that *Tropilaelaps* mites in China were distinct from the *T. clareae*, *T. koenigerum*, and *T. thaii*.

3.3 Restriction enzyme markers for identifying *Tropilaelaps* mites

The particular restriction sites surveyed here were selected because previous studies have shown no variation within each of the four *Tropilaelaps* species, but reliable differences

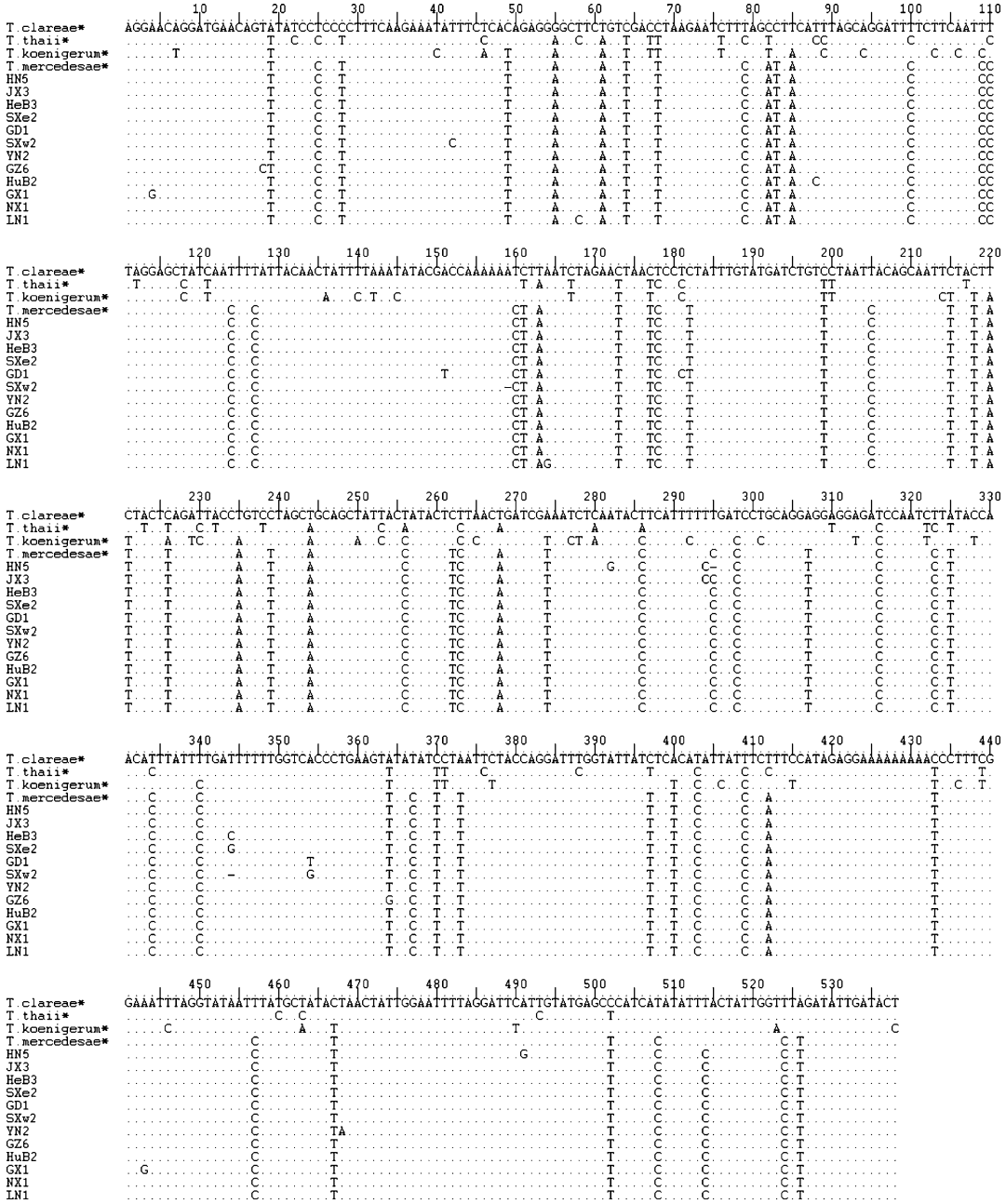

Figure 2. Alignment of mtDNA *cox1* sequences of representative *Tropilaelaps* mites in this study. The reference sequences are indicated by an *asterisk* (*). A *dot* indicates an identical nucleotide and hyphens refer to insertion/deletion. The GenBank accession no. of sequences in this study were HQ533148 (HN5), HQ533149 (JX3), HQ533150(HeB3), HQ533151 (SXe2), HQ533152 (GD1), HQ533153 (SXw2), HQ533154 (YN2), HQ533155 (GZ6), HQ533156 (HuB2), HQ533157 (GX1), HQ533158 (NX1), and HQ533159 (LN1).

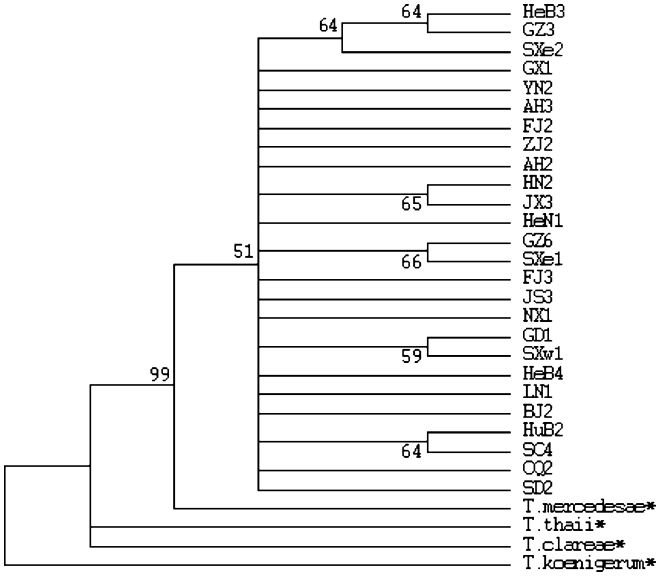

Figure 3. A neighbor-joining phenogram illustrating relationships between representative *Tropilaelaps* mites in this study and other reference *Tropilaelaps* species based on mitochondrial *cox1* sequence. The reference *Tropilaelaps* species are indicated by an *asterisk* (*).

among species. Restriction enzyme analyses on 429 mites from each location in this study confirmed that mite samples from different locations were invariant. All samples showed typical restriction patterns of the *T. mercedesae*: the *Bsr*I site in *cox1* and the *Rsa*I site in ITS1-5.8S-ITS2 were present, and the *Swa*I, *Fau*I, *Bst*YI sites in *cox1* was absent.

4. DISCUSSION

Based on the sequence variation or specific restriction sites in *cox1* and ITS1-5.8S-ITS2 fragments, all *Tropilaelaps* mites examined in this study were shown to represent *T. mercedesae*, which has been mistaken for *T. clareae* until now. Thus, our results clarify the taxonomic status of *Tropilaelaps* mites infesting *A. mellifera* in China.

These results confirm a similar finding by Anderson and Morgan (2007). We did not detect *T. clareae*, *T. koenigerum*, and *T. thaii* infesting *A. mellifera* in China. This may be explained by the fact that none of these mite species has been detected in *A. dorsata* in China (Anderson and Morgan 2007), which is the natural host of *Tropilaelaps* mites. Thus, in China, only the species *T. mercedesae* may have been available to transfer to *A. mellifera* as an alternate host.

More recently, the health of honeybee is drawing special attention because of reported declines in bee colonies and the "ecosystem services" they provide. This issue has been brought to even more attention by recent devastating losses of honey bees throughout North America (so called, "Colony Collapse Disorder"; Otterstatter and Thomson 2008; vanEngelsdorp et al. 2009); yet, we still have little understanding of the cause(s) of bee declines. Although there were no reports of high losses in China, honeybee health has gained special attention in the past 3 years. Although *Tropilaelaps* is one of the major threats for the health of *A. mellifera* in Asia, it has received comparatively little scientific interest to date and basic studies are still lacking. Several areas of research should be addressed. For example, given the lack of samples from *A. dorsata* in this study, maybe other *Tropilaelaps* haplotypes or species await discovery in *A.*

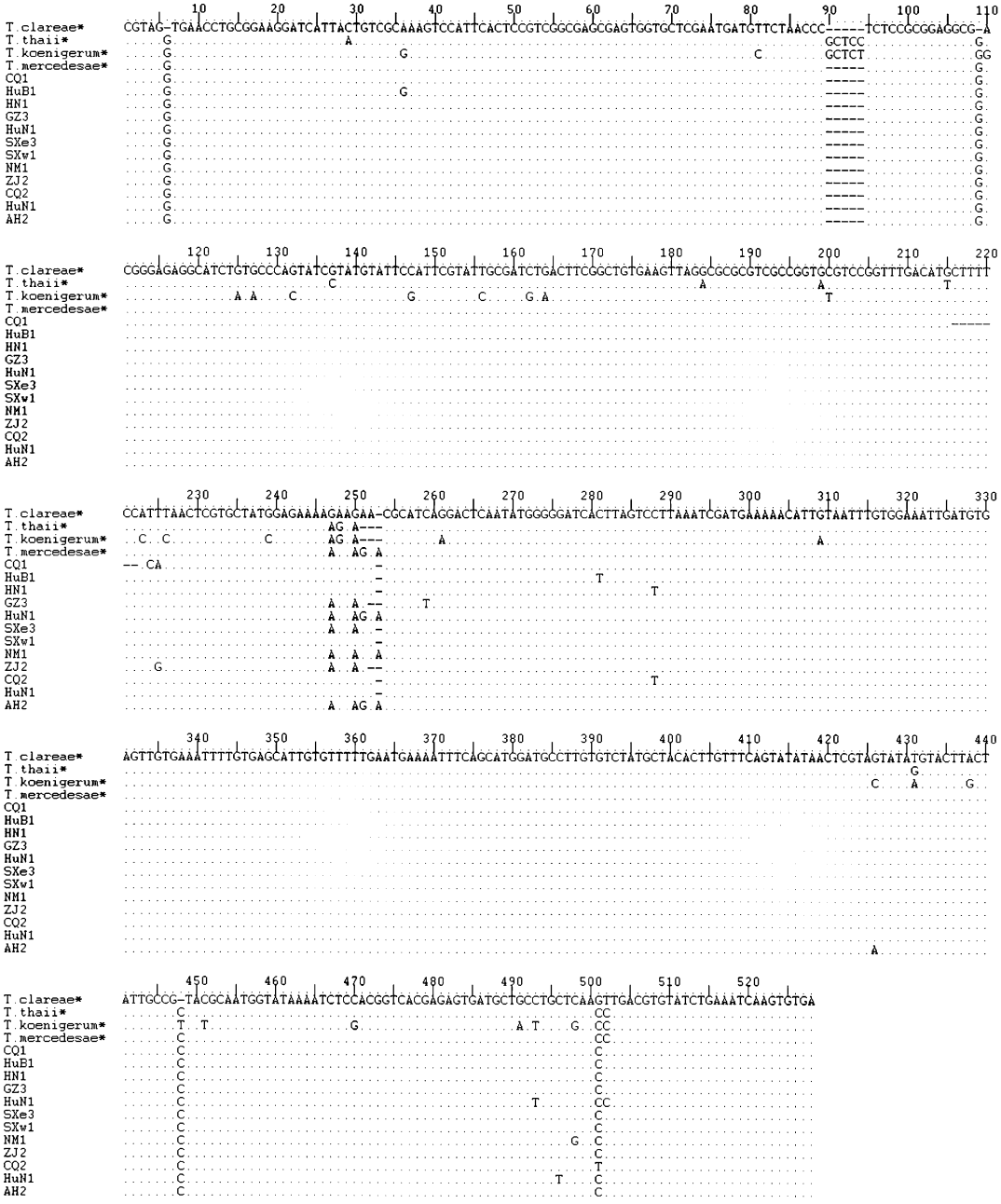

Figure 4. Alignment of ITS1-5.8S-ITS2 sequences of representative *Tropilaelaps* mites in this study. The reference sequences are indicated by an *asterisk* (*). A *dot* indicates an identical nucleotide and hyphens refer to insertion/deletion. The GenBank accession no. of sequences in this study were HQ533160(CQ1), HQ533161 (HuB1), HQ533162(HN1), HQ533163(GZ3), HQ533164(HuN1), HQ533165(SXe3), HQ533166(SXw1), HQ533167(NM1), HQ533168(ZJ2), HQ533169(CQ2), HQ533170(HuN1), and HQ533171(AH2).

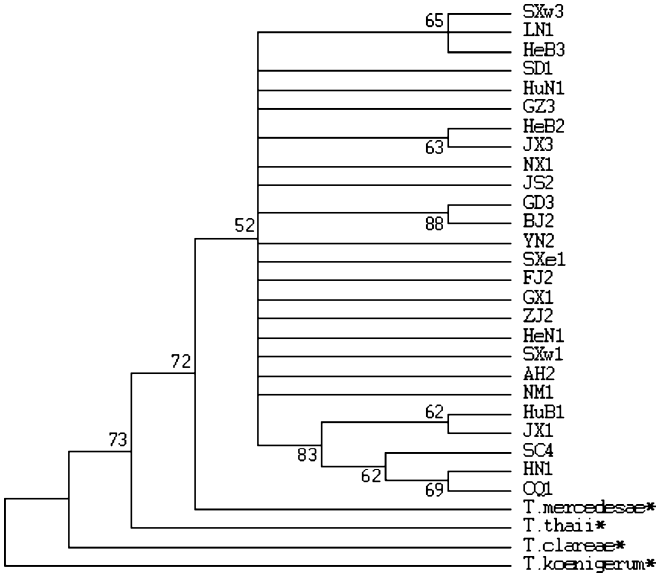

Figure 5. A neighbor-joining phenogram illustrating relationships between representative *Tropilaelaps* mites in this study and other reference *Tropilaelaps* species based on ITS1-5.8S-ITS2 sequence. The reference *Tropilaelaps* species are indicated by an *asterisk* (*).

dorsata in China. Also, the relationship among *Tropilaelaps* mites, *A. dorsata* and *A. mellifera* is not clear, and how *Tropilaelaps* actually damages these hosts. Next, we need to determine whether any of these newly described *Tropilaelaps* species will also cross-infest the endemic domesticated honeybee, *A. cerana*. Then, epidemiological studies to identify relevant and potentially avoidable exposures are very important. Finally, additional research on in vitro culture of *Tropilaelaps* mites is needed, although laboratory methods for culturing *T. clareae* were developed by Rath (1991).

ACKNOWLEDGMENTS

This study was supported by grants from National Natural Science Foundation of China (No. 30871831). We would like to thank the editors and two anonymous referees for detailed comments and suggestions. Thanks are also due to Dr. Jeff Pettis from the USDA-ARS Bee Research Laboratory for comments on the manuscript. Special thanks go to Jun Guo (Institute of Bees Research, Chongqing Academy of Animal Sciences), who have helped us collect samples in this study.

Identification des acariens du genre *Tropilaelaps* (Acari, Laelapidae) infestant *Apis mellifera* en Chine.

***Tropilaelaps mercedesae* / identification / *Apis mellifera* / Chine / mtDNA / cox1**

Die Identifizierung von *Tropilaelaps* Milben (Acari, Laelapidae) auf *Apis mellifera* in China

***Tropilaelaps mercedesae* / Identifizierung / *Apis mellifera* / mtDNA / *cox1* / ITS1-5.8S-ITS2 / China**

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool, J. Mol. Biol. **215**, 403–410.

Anderson, D.L., Gibbs, A.J., Gibson, N.L. (1998) Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences, Mycol. Res. **102**, 541–547.

Anderson, D.L., Morgan, M.J. (2007) Genetic and morphological variation of bee-parasitic Tropilaelaps mites (Acari: Laelapidae): new and re-defined species, Exp. Appl. Acarol. **43**, 1–24.

Bailey, L., Ball, B.V. (1991) Honey bee pathology, Academic Press, London.

Brown, W.M., George, M.J., Wilson, A.C. (1979) Rapid evolution of animal mitochondrial DNA, Proc. Natl. Acad. Sci. U.S.A. **76**, 1967–1971.

Burgett, M., Akratanakul, P., Morse, R.A. (1983) *Tropilaelaps clareae*: A parasite of honeybees in South-East Asia, Bee World **64**, 25–28.

de Jong, D., Morse, R.A., Eickwort, G.C. (1982) Mite pests of honeybees, Annu. Rev. Entomol. **27**, 229–252.

Delfinado, M.D., Baker, E.W. (1961) Tropilaelaps, a new genus of mite from the Philippines (Laelapidae, Acarina), Fieldiana Zool. **44**, 53–56.

Efron, B., Halloran, E., Holmes, S. (1996) Bootstrap confidence levels for phylogenetic trees, Proc. Natl. Acad. Sci. U.S.A. **93**, 7085–7090.

Garnery, L., Solignac, M., Celebrano, G., Cornuet, J.M. (1993) A simple test using restricted PCR amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L., Experientia **49**, 1016–1021.

Hillis, D.M., Bull, J.J. (1993) An empirical-test of bootstrapping as a method for assessing confidence in phylogenetic analysis, Syst. Biol. **42**, 182–192.

Hillis, D.M., Davis, S.K. (1986) Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus, Rana, Evolution **40**, 1275–1288.

Hillis, D.M., Dixon, M.T. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference, Q. Rev. Biol. **66**, 411–453.

Kumar, S., Tamura, K., Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment, Brief Bioinform **5**, 150–163.

Laigo, F.M., Morse, R.A. (1968) The mite *Tropilaelaps clareae* in Apis dorsata colonies in the Philippines, Bee World **49**, 116–118.

Laigo, F.M., Morse, R.E. (1969) Control of the bee mites Varroa jacobsoni Oudemans and *Tropilaelaps clareae* Delfinado and Baker with chlorbenzilate, Philippine Entomol. **1**, 144–148.

Luo, Q.H., Peng, W.J., An, J.D., Guo, J. (2008) The potential causes of colony collapse disorder (CCD) and its countermeasures in China, Chin. Bull. entomol. **45**, 991–995.

Mindell, D.P., Sorenson, M.D., Huddleston, C.J., Miranda, H.C., Knight, A., et al. (1997) Phylogenetic relationships among and within select avian orders based on mitochondrial DNA, in: Mindell D.P. (Ed.), Avian molecular evolution and systematics, Academic Press, New York, pp. 214–217.

Moore, W.S. (1995) Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees, Evolution **49**, 718–726.

Odorico, D.M., Miller, D.J. (1997) Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of Acropora (Cnidaria; Scleractinia): patterns of variation consistent with reticulate evolution, Mol. Biol. Evol. 14, 465—473.

Otterstatter, M.C., Thomson, J.D. (2008) Does pathogen spillover from commercially reared bumble bees threaten wild pollinators, PLoS ONE 3, e2771.

Rath, W. (1991) The laboratory culture of the mites Varroa jacobsoni and *Tropilaelaps clareae*, Exp. Appl. Acarol. **10**, 289–293.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods, Bioinformatics **19**, 2496–2497.

Saiki, R.K. (1990) Amplification of genomic DNA, in: Innis M.A., Gelefland D.H., Snensky J.J., White T.J. (Eds.), PCR Protocols: methods and applications, Academic Press, San Diego, CA, pp. 13–20.

Schmid-Hempel, P. (1998) Parasites in social insects, Princeton, New Jersey.

Tangjingjai, W., Verakalasa, P., Sittipraneed, S., Klinbunga, S., Lekprayoon, C. (2003) Genetic differences between *Tropilaelaps clareae* and *Tropilaelaps koenigerum* in Thailand based on ITS and RAPD analyses, Apidologie **34**, 514–524.

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

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., et al. (2009) Colony Collapse Disorder: a descriptive study, PLoS ONE 4, e6481.

White, T.J., Bruns, T., Lee, S., Tayler, J. (1990) Ampliffication and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (Eds.), PCR protocols, Academic Press, San Diego, pp. 315–322.

Woyke, J. (1994) Mating behaviour of the parasitic honeybee mite *Tropilaelaps clareae*, Exp. Appl. Acarol. **18**, 723–733.

Zeng, Z.J. (2003) Discussion about the hosts of T. clareae, Journal of bee **56**, 40–41.