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Genetic Profile of the Parasitic Varroan Mite *Varroa destructor* (Arachnida: Mesostigmata: Varroidae) in Taiwan: a New Taiwanese Haplotype Intermediate Between the Highly Virulent Russian and Less Virulent Japanese Types Identified in the Honey Bee Host *Apis cerana*

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The Modern beekeeping industry is being challenged by the varroan mite and its transmitted pathogens. Various types of *Varroa destructor* exhibit different levels of virulence toward honey bees, but only the Japanese (J) and Russian (R) types were found to infect *Apis mellifera*. Type R was more highly virulent against *A. mellifera* in comparison with type J. Examining the genetic profile of Varroa species is therefore of crucial importance in apiary management. In this study, maternally inherited mitochondrial cytochrome oxidase I (*COI*) and bisexual nuclear internal transcribed spacer (ITS) sequences of *V. destructor* individuals from Taiwan were determined. All 168 *COI* sequences observed in populations obtained from *A. mellifera* were identical and belonged to type J, with one base difference to that of populations collected from *A. cerana*; the new type is named 'T type' (Taiwan type). ITS sequences of *V. destructor* and its sister species *V. jacobsoni* were identical. A network analysis based on 611 *COI* sequences compiled from references indicated the presence of 27 haplotypes in *V. destructor*. Epidemic history and relationship analyses of *V. destructor* showed that the basal haplotypes were those from *A. cerana* and many R-extending haplotypes infesting *A. mellifera* involving amino acid substitutions. Calibration dating based on *COI* analysis revealed that *V. destructor* differentiated from its sibling lineage (occurring in Sri Lanka) prior to 1.3 million years ago (Mya). The ancestral haplotype retention and drift in *V. destructor* that occurred locally during 0.10–0.64 Mya might be relevant to its host *A. cerana*, which had been isolated geologically. The highly virulent type R was spreading quickly and could gradually outcompete the common and less virulent type J. Type T, being intermediate between types R and J, ought to be studied to better understand the pathogenic mechanism of *V. destructor* in *A. mellifera*. Moreover, for areas where type R does not occur, such as Taiwan, quarantine requirements are crucial for reducing invasion risks.

Key words: Mite, *Varroa destructor*, Bee, *COI*, ITS.

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BACKGROUND

Colony Collapse Disorder has considerably reduced the honey production and pollination efficiency of honey bees worldwide over the past three decades. Multiple factors can drive honey bee colony losses, the parasitic varroan mites and their transmitted viruses being among the most significant ones, exerting a substantial adverse effect on the modern beekeeping industry (Rosenkranz et al. 2010; Mondet et al. 2014; McMenamin and Genersch 2015). Four *Varroa* species have been described, namely *Varroa destructor* Anderson and Trueman, *Varroa jacobsoni* Oudemans, *Varroa rindereri* de Guzman and Delfinado-Baker, and *Varroa underwoodi* Delfinado-Baker and Aggarwal. Each varroan species has its *Apis* host (de Guzman and Delfinado-Baker 1996; de Guzman and Rinderer 1999; Wang et al. 2019); however, only *V. destructor* infects *Apis mellifera* (Anderson and Trueman 2000).

For a long time, *Varroa destructor* was considered a biotype of *V. jacobsoni* until Anderson and Trueman (2000) described it as a distinct species. The varroan species that infects *A. mellifera* is larger than the one that infects *A. cerana*, and it was regarded as a distinct biotype with different levels of virulence toward these two bee species (Delfinado-Baker 1988; Delfinado-Baker and Houck 1989). Currently, we know that biotype A (also known as the Russian, German, or Korean type) is a group of *V. destructor* that infects *A. mellifera* and frequently causes its death. Biotype B is a group of *V. jacobsoni* that infects several *Apis* species but generally does not cause their death. Biotype C (also known as the Japanese type) is a group of *V. destructor* that infects *A. mellifera* but does not cause its death (Delfinado-Baker 1988; Delfinado-Baker and Houck 1989; Anderson and Trueman 2000). The body size and shape of *V. jacobsoni* and *V. destructor* are different; their size ratio is ≥ 1.4 (Dietemann et al. 2013). Cytochrome oxidase I (*COI*) sequences are different between *V. jacobsoni* and *V. destructor* (Anderson 2000; Anderson and Trueman 2000).

Kraus and Hunt (1995) studied the genetic variability of *V. destructor* using randomly amplified polymorphic DNA (RAPD) analysis and reported that the *Varroa* species occurring in the United States has the same RAPD pattern as the one found in Germany. Based on a RAPD analysis, de Guzman et al. (1997) proposed that Eastern Russia and Japan might have been the original territories of the *V. destructor* infecting *A. mellifera*. Subsequent genetic studies have revealed that the RAPD pattern is consistent in identification with *COI* divergences (de Guzman et al. 1998; Anderson and Fuchs 1998; de Guzman and Rinderer 1999; de Guzman et al. 1999; Anderson and Trueman 2000).

Anderson and Trueman (2000) recognized *V. destructor* and *V. jacobsoni* as two different species based on mitochondrial *COI* sequences and morphological characters, and revealed that *V. destructor* but not *V. jacobsoni* is harmful to *A. mellifera*; the divergence of their *COI* sequences is approximately 7%. In subsequent studies, *COI* sequences were extensively used for identification of these two species of *Varroa*. It has been established that *V. jacobsoni* distributed in Malaysia and Indonesia infects several *Apis* species but does not reproduce in *A. mellifera*, whereas *V. destructor* infects both *A. cerana* and *A. mellifera*. Before the introduction of the Western honey bee into East Asia, *V. destructor* was considered to infect only *A. cerana*, a bee species distributed in South and East Asia, including Pakistan, Nepal, India, Sri Lanka, northern Thailand, Vietnam, China, Korea, the Russian Far East, Japan, and Taiwan (Crane 1978; de Guzman et al. 1999; Anderson and Trueman 2000; Solignac et al. 2005). Based on the presence of sympatric populations of *A. mellifera* and *A. cerana*, two independent host switch events, one in Eastern Russia and the other in Japan in the 1950s, were proposed by Crane (1984) and de Guzman et al. (1997), respectively. The Russian type was probably first introduced to eastern Europe in the 1960s via transported brood combs. Subsequently, it spread to Germany and other parts of Europe, later establishing itself worldwide and becoming the main pest that damages *A. mellifera* (Crane 1978; de Guzman et al. 1997; Rosenkranz et al. 2010). *Varroa destructor* has been known to infest *A. cerana* in Japan since 1909, but had not been detected in *A. mellifera* in Japan until 1957 (Crane 1984). The Japanese type may have been exported to Taiwan and southeastern Asia during 1972 (Lo and Chao 1975). It was transported to South America in the 1970s and then to the United States in the late 1990s (de Jong and Goncalves 1981; de Jong et al. 1982; Delfinado-Baker and Houch 1989; de Guzman et al. 1997 1999).

A comprehensive sampling by Anderson and Trueman (2000) enabled haplotype identification of *V. destructor* based on the mitochondrial *COI* amplicon. Each haplotype was assigned the name of the area (country or island) in which it was discovered in *A. cerana*. Of their six defined haplotypes (Nepal, Sri Lanka, Vietnam, China, Japan/Thailand, and Korea), only the Japanese and Korean haplotypes were found to infect *A. mellifera*. The Korean type was found to be identical with the Russian type (Anderson 2000), but in the present study, the Japanese (J) and Russian (R) types are used to represent haplotypes of the defined types of Japan/Thailand and Korea, respectively, in accordance with the original area of the relevant host-shift event (Crane 1978 1984) and the priorities of synonyms

indicated by de Guzman et al. (1997) and Anderson (2000). A new, more complex haplotype naming system, based on newly available sequences of *COI* and other amplicons, *e.g.*, "pure J", "J1-2" or "J-like", has been proposed (Solignac et al. 2005; Navajas et al. 2010; Traynor et al. 2020).

Although the reasons why only types J and R are capable of infecting *A. mellifera* have remained unclear, type R has been found to be highly virulent toward *A. mellifera* and it can outcompete type J in localities where they are sympatric (de Guzman et al. 1997; Anderson 2000; Garrido et al. 2003; Strapazzon et al. 2009; Dietemann et al. 2019). By contrast, type J is considerably less aggressive and it does not cause mortality (Delfinado-Baker 1988; de Guzman et al. 1998; de Guzman and Rinderer 1999; Carneiro et al. 2007). Colonies of type R were found to be more abundant than type J in sympatric areas (de Guzman et al. 1999; Carneiro et al. 2007). A naturally *Varroa*-resistant population of *A. mellifera* has also been observed worldwide (Locke 2016; Mondet et al. 2020). Microsatellite analysis indicated that hybridization events were not rare in areas where both types R and J occurred (Solignac et al. 2005; Dietemann et al. 2019) without the presence of any particular asymmetrical introgression, suggesting a postzygotic isolation between the two types.

It is important to identify and elucidate haplotypes of *Varroa* mites since their identification is the basis for species management, which is necessary to protect the bee industry from an invasion of the highly virulent type R. Additionally, the identification of new haplotypes infesting *A. cerana* is highly significant, because such haplotypes pose a potential threat to *A. mellifera*, as it might become a new host in the future (Warrit et al. 2006; Navajas et al. 2010; Dietemann et al. 2019; Wang et al. 2019; Traynor et al. 2020). Thus, the haplotype profiles of *V. destructor* documented in Argentina, Brazil, China, Madagascar, Mexico, Spain, and Thailand in the last two decades are critical for inferring the evolutionary history of the species and developing management strategies against it (Medina and Martin 1999; Warrit et al. 2006; Muñoz et al. 2008; Navajas et al. 2010; Maggi et al. 2012; Rasolofoarivao et al. 2013; Octaviano-Salvadé et al. 2017; Dietemann et al. 2019; Wang et al. 2019; Traynor et al. 2020).

Apis cerana started being cultured mainly in the southwestern areas of Taiwan more than 300 years ago, while *A. mellifera* was imported to the island in 1910 from the United States (Himura 1912; Inoue 1913; Hong 1962). *Varroa jacobsoni* was first recorded in 1975 in Taiwan, but the population was later identified as *V. destructor* (Lo and Chao 1975). The present study is based on a survey of *V. destructor* conducted throughout Taiwan between 2017 and 2020. A total of 14 *Varroa* colonies from *A. mellifera* and *A. cerana* in Taiwan, China, and Thailand were collected and their morphological and molecular characters were evaluated. The 211 sequences acquired in this study were analyzed in combination with 400 additional available *COI* sequences of *V. destructor*. Moreover, bisexual nuclear internal transcribed spacer (ITS) sequences of *V. destructor* were determined. Similar to hybrid detection using microsatellite markers, the identification of ITS sequences can be helpful for evaluating gene flow phenomena or hybridization events between various *Varroa* lineages (Solignac et al. 2005; Dietemann et al. 2019). Findings regarding haplotype profile and globally differentiated pattern can be informative for the development of *V. destructor* management strategies as well as quarantine requirement considerations, particularly in areas without severe damage by type R.

MATERIALS AND METHODS

Sample collection

Adult females of *V. destructor* were collected from the bee brood cells of *A. mellifera* colonies in 12 apiaries across Taiwan including 2 populations of *A. cerana* (Fig. S1). During the autumns of 2017 to 2020, 182 *Varroa* mites (5–25 individuals from each apiary) were analyzed (Table S1). Moreover, 8 and 30 samples from *A. mellifera* apiaries in China (Jilin Province) and Thailand (Chiang Mai), respectively, were employed to determine the *COI* and ITS sequence variation among populations.

Morphological description of Taiwanese *V. destructor*

Measurements of body widths and lengths of 31, 3, and 23 individuals of *Varroa* mites collected from *A. mellifera* in Taiwan, China, and Thailand, respectively, as well as 5 individuals from *A. cerana* in Taiwan, were taken using a Microsight 4.1.2 (Q-Optics, USA) connected to a Canon EOS 800D camera (Tokyo, Japan) (Fig. 1). Specimen images were captured using a Nikon Coolpix B700 camera (Tokyo, Japan) with a Raynox DCR-250 macrolens (Tokyo, Japan). A statistical analysis of the measurements was carried out using the R software, following statistics proceedings described by R Development Core Team (R version 4.1.2). Analysis of variance (ANOVA) was used to test the measured variables for the aforementioned 4 varroan groups. The nonparametric Kruskal-Wallis test followed by Wilcoxon rank sum test for post hoc comparison

($p < 0.05$) were applied for the significant test of size measurement (Valdano and Di Rienzo 2007).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from adult female *V. destructor* individuals by using a Quick Extract DNA extraction kit (Epicentre Biotechnologies, Madison, WI). Primer sets were used to amplify the mitochondrial *COI* gene and nuclear ITS region. The sequences of forward and reverse primers for *COI* amplification, WX-990422 and ZSF-20060123, respectively, were reported by Wang (2007). Forward and reverse primers for the ITS region, namely ITSvarroa1 (TGARMCTGCGGARGGAWCATTAC) and ITSvarroa2 (CACACTTGATTTCAGATAAACATAAC), respectively, were designed in this study on the basis of the acquired *Varroa* mite sequences and conserved regions of ribosomal 18S and 28S DNA sequences (Kjer et al. 1994). Polymerase chain reaction (PCR) was performed in a volume of 25 µl containing 0.5 µl of each primer (10 mM), 0.2 µl of each dNTP (25 mM), 2.5 µl 10X Taq buffer, 0.5 µl Super Taq polymerase, and 1 µl of DNA template. The PCR programming conditions were as follows: 94°C for 2 min for denaturation, followed by 35 cycles at 94°C for 50 s, 42°C for 50 s, and 72°C for 50 s. The final extension was 72°C for 10 min. The PCR products were purified from 1% agarose gel by using the QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) and then sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic and sequence analyses

The sequences of forward and reverse strands of each specimen were piled up with the published *COI* sequences of *V. destructor* and then aligned using ClustalW multiple alignment in Bioedit 7.0 (Hall 1999). In total, 211 *COI* sequences were acquired in this study, 168, 25, and 3 of which were collected from *A. mellifera* from Taiwan, Thailand, and China, respectively; and 15 were from Taiwanese *A. cerana*. Moreover, the 400 *COI* sequences of *V. destructor* obtained from GenBank and data in prominent references were compiled to elucidate the *COI* divergence (Table S2 and Fig. S2). The putative amino acid sequence was translated from the *COI* sequences and the pairwise variation of sequences was estimated based on the uncorrected proportional divergence by using MEGAX software (Kumar et al. 2018). The *COI* sequence divergences were also compared among *V. destructor*, *V. jacobsoni* (AF107902-AF107910), *V. underwoodi* (AF107260), and *V. rindereri* (AF107261). Phylogenetic analysis based on the *COI* haplotype sequences of *V. destructor* and *V. jacobsoni* was performed using Bayesian inference. The best-fit substitution model for *COI* was analyzed in jModelTest 0.1 by using the Bayesian information criterion (BIC) and was discovered to be GTR+I+G in MrBayes version 3.2 (Posada 2008; Ronquist et al. 2012). Three hot chains and one cold chain were applied for 1×10^8 generations with sampling every 1000 generations (Ronquist et al. 2012). The analysis was halted when the average standard deviation of the split frequencies was less than 0.002.

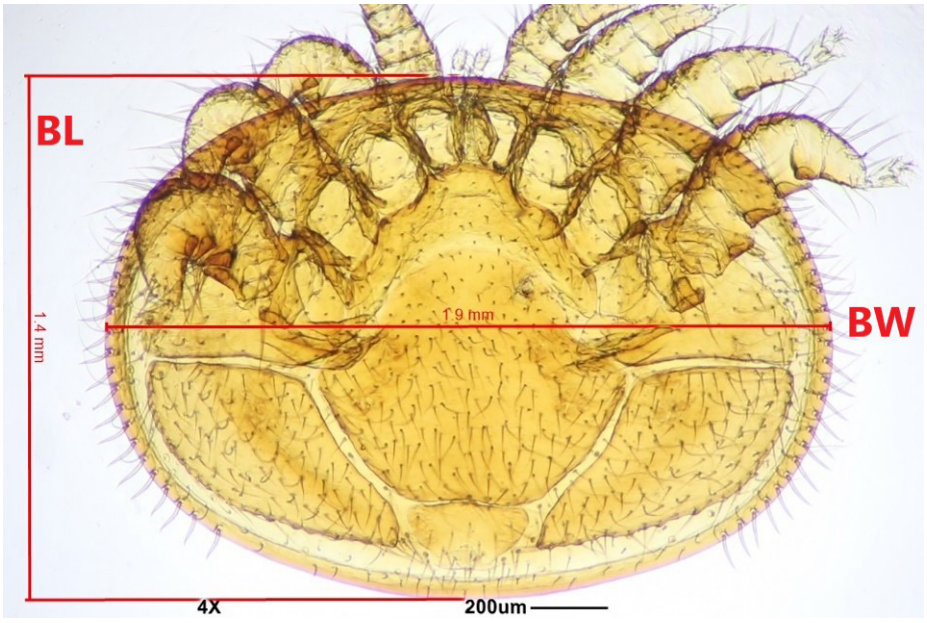

Fig. 1. Morphological measurements of Taiwanese *Varroa destructor.* BL: body length, BW: body width. Scale bar in 200 µm was shown.

The first 25% of trees were discarded as burn-in, and the remaining 75% of trees were used to construct a consensus tree.

Haplotype separation was conducted using DNASP software (Rozas et al. 2003). Haplotype networks were analyzed using TCS version 1.21 with a statistical parsimony criterion (Clement et al. 2000). Based on the phylogenetic inferences, the most divergent type of *V. destructor*, namely the Sri Lankan type, was used for the outgroup comparisons in the network analysis. Moreover, DELTRAN optimization was applied in the haplotype network to determine the evolved processes of ancestral and derived haplotypes (Agnarsson and Miller 2008). This is because the most recent mutation of *V. destructor* in the new host, *A. mellifera*, is likely to occur as far as possible towards the tips in the network process.

Calibration dating for *COI* sequences

The divergence time of *V. destructor* haplotypes was estimated with the help of BEAST 2.6 (Bouchaert et al. 2014) by using the closely related haplotypes of Luzon 1 and Luzon 2, and those of *V. jacobsoni* as outgroups (Anderson and Trueman 2000). The best-fit model for nucleotide substitution was determined in jModelTest 0.1 by using the BIC (Posada 2008). GTR was the suitable model obtained in BEAST version 2.6 for *COI* sequences. Two substitution rates of 1.5% and 1.15% per lineage over a million years for *COI* sequences, commonly used for insects, were applied separately in this study (Brower 1994; Papadopoulou et al. 2010). A Markov chain Monte Carlo analysis was run for 1×10^8 generations with sampling performed every 1×10^3 generation. The effective sample size for the posterior distribution of estimated parameter values was analyzed using Tracer version 1.5 until the suggested value was reached (*i.e.*, 200) (Rambaut and Drummond 2009). Subsequently, the initial 25% of the run was discarded as burn-in (Rambaut and Drummond 2009).

RESULTS

Morphological measurements of the *V. destructor* population in Taiwan

Body lengths and widths of *V. destructor* specimens collected from *A. mellifera* in three different countries and from *A. cerana* in Taiwan are shown in table 1. Although a similar body length of 1200 m exists among the varroan mites, the body width is found to be significantly different in Taiwanese and Thai populations based on the nonparametric Kurskal-Wallis test.

COI and ITS sequence variations of *V. destructor* populations in Taiwan compared with other varroan species

The *COI* and ITS sequences of 458 bp and 516 bp, respectively, were successfully amplified. *COI* sequences of 196 and 15 specimens of *V. destructor* obtained from *A. mellifera* and *A. cerana*, respectively, were determined. *COI* and ITS sequences of *V. destructor* were deposited into the NCBI GenBank under accession numbers OK335527-OK35737 and OK336567-OK336693, respectively. The other two ITS sequences of Thai *V. jacobsoni* were deposited under accession number OK335749-OK335750. Individuals of *V. destructor* from 12 colonies across Taiwan were found to have identical *COI* and ITS amplicons except for a single base difference in the *COI* sequence in samples taken from *A. cerana*. The average genetic distances in the *COI* sequences between *V. destructor* samples from Taiwan in relation to *V. underwoodi*, *V. rindereri*, *V. jacobsoni*, the Mindanao varroan type, and the Luzon varroan type were found to be 9.0%, 8.6%, 6.6%, 4.8%, and 4.2%, respectively. In the ITS region, the average proportions in T, C, A, and G in the *V. destructor* specimens were 34.4%, 13.5%, 29.1%, 23.0%, respectively, and no sequence variation was observed between *V. destructor* and *V. jacobsoni*

Table 1. Mean and stand deviation (SD) of body size measurement (mm) for *Varroa destructor* populations from Taiwan, Thailand, and China

Measurement Population	Body Length* Mean ± SD	Body Width* Mean ± SD	Ind. No[1]	Host[2]
Taiwan	1.201 ± 0.051^{a}	1.720 ± 0.053^{b}	31	A. mel
China	1.259 ± 0.126^{a}	1.783 ± 0.029^{b}	3	A. mel
Thailand	1.201 ± 0.061^{a}	1.695 ± 0.061^{c}	23	A. mel
Taiwan	1.288 ± 0.071^{a}	1.836 ± 0.058^{a}	5	A. cer

*Significant divergence was determined by Kruskal-Wallis test followed by Wilcoxon rank sum test for post hoc significance ($p < 0.05$). Different letters mean significantly divergent. [1]Number of individuals measured. [2]A. mel: *Apis mellifera*; A. cer: *Apis cerana*.

(EF025475 and two Thai individuals of OK335749-OK335750).

Relationship of *V. destructor* haplotypes

Twenty-seven haplotypes were identified among the 611 *COI* sequences obtained from *V. destructor*, among them six haplotypes previously identified by Anderson and Trueman (2000). All *V. destructor COI* from *A. mellifera* in Taiwan belonged to type J; one new haplotype, named here as the Taiwan type (type T), was found in Taiwanese *A. cerana*. A network analysis revealed a detailed evolved pattern among haplotypes (Fig. 2A). The Sri Lankan haplotype used as outgroup had at least 13 substitution steps to other haplotypes, and substitution steps ranged from 1 to 9 among the remaining 26 haplotypes, except the peculiar type MK482687 from Iran. The network originating from the sibling Sri Lankan haplotype is closely connected to the haplotypes of China3 and J. The newly identified type T from Taiwanese *A. cerana* is intermediate between types J and R. The network also shows that 13 out of 16 R-derived types found in tropical areas of Asia, *i.e.*, southern India, Iran, and Saudi Arabia, involved amino acid substitutions (Fig. 3). Type R is found to be distributed worldwide except in Australia, Taiwan, and Puerto Rico (Table S2). The Vietnamese haplotype was also observed in China and Thailand.

A Bayesian analysis conducted on *COI* haplotype sequences revealed that the lineages of Luzon 1 and Luzon 2 are distributed between *V. jacobsoni* and *V. destructor* (Fig. S3). In *V. destructor*, the Sri Lanka type is the most basal, followed by the types of J and Nepal. The remaining types either come from *A. cerana* (China, Vietnam, Taiwan, and Russia) or from *A. mellifera*, exhibiting a polytomous relationship (Fig. S3).

Calibration dating of *V. destructor*

Although a polytomous relationship is found among the shallow divergent haplotypes, calibration dating based on the *COI* haplotype sequences of *V. destructor* and *V. jacobsoni* revealed that the types from Nepal, Japan, Vietnam, and China found in *A. cerana* are grouped in the same cluster, whereas those found in *A. mellifera* in India, Iran, and Saudi Arabia are grouped with types R and T (Fig. 4). Calibration dating revealed that the lineage of *V. destructor* diverged from that

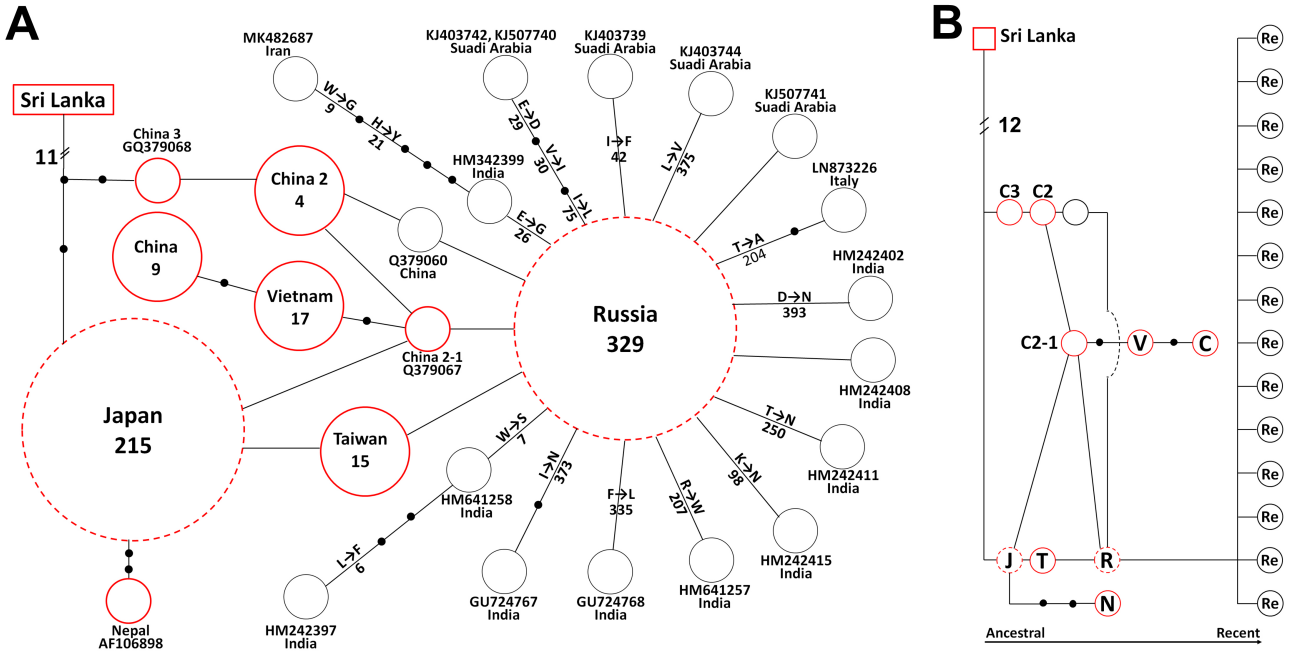

Fig. 2. Haplotype network (A) and the possible evolved processes of ancestral and derived haplotypes according to the DELTRAN optimization (B) of *Varroa destructor* based on the *COI* haplotype sequences. The haplotype of Sri Lanka in the rectangle was used as the outgroup. Red and black circles represent the sampling mites parasitized in the host of *Apis cerana* and *A. mellifera*, respectively. The dotted red circles of Japanese and Russian haplotypes have both hosts of *A. cerana* and *A. mellifera*. A hypothetical haplotype is shown in a dot black circle. (A) The small circle corresponds to one individual, and the individual's number of remaining haplotypes is marked within the corresponding medium and large circles. The nonsynonymous substitution position and its inducing amino acid changes between haplotypes are labeled. (B) The abbreviations of the haplotype are shown based on the haplotype name on panel A and Re means the extending haplotype from the Russian haplotype. C, C2, C2-1, C3, J, N, R, T, and V indicate the haplotypes of China, China 2, China 2-1, China 3, Japan, Nepal, Russia, Taiwan, and Vietnam, respectively.

A

	1111 1122222222 3333333344 444
	12222344 5678890244 5600244579 1123677923 355
	8911389027 9853681836 8747425023 1435835384 725
Sri Lanka	TTACTAAGAA ATAGAGATTA TAACAGTCAA TGATTTTGTT TGA
Nepal	....C....G TA.A..GCCT ....GA...T ....A. CAG
China	....C....G T..AG.GC.T ....T CA......A. CAG
China 2	....C....G T..A..GCCT ....T C.......G. CAG
China 2-1	....C....G T..A..GCCT ....T C.......A. CAG
China 3	....C....G T..A..GC.T ....T C.......G. CAG
Vietnam	....C....G T..AG.GCCT ....T C.......A. CAG
Taiwan	....C....G T..A..GCCT .G.......T ....A. CAG
Japan	....C....G T..A..GCCT ....T ....A. CAG
Russia	....C....G T..A..GCCT .G.......T C.......A. CAG
GQ379060_China	....C....G T..A..GCCT .G.......T C.......G. CAG
GU724767_India	....C....G T..A..GCCT .G.......T C...G*A*..A. CAG
GU724768_India	....C....G T..A..GCCT .G.......T C..*A*....A. CAG
HM242397_India	*AA*G.C....G T..A..GCCT .G....C..T C.......A. CAG
HM242399_India	....C*G*...G T..A..GCCT .G.......T C.......A. CAG
HM242402_India	....C....G T..A..GCCT .G.......T C......*A*A. CAG
HM242408_India	....C....G T..A..GCCT *C*G.......T C.......A. CAG
HM242411_India	....C....G T..A..GCCT .G.....*A*.T C.......A. CAG
HM242415_India	....C....G T..A.CGCCT .G.......T C.......A. CAG
HM641257_India	....C....G T..A..GCCT .G.*T*.....T C.......A. CAG
HM641258_India	.*A*..C....G T..A..GCCT .G.......T C.......A. CAG
KJ403739_Saudi Arabia	....C...*T*G T..A..GCCT .G.......T C.......A. CAG
KJ403744_Saudi Arabia	....C....G T..A..GCCT .G.......T C.....*G*.A. CAG
KJ403742_Saudi Arabia	....C.*TA*.G T.*C*A..GCCT .G.......T C.......A. CAG
KJ507741_Saudi Arabia	....C....G T..A..GCCT .G.......T C.......AC CAG
LN873226_Italy	....C....G TC.A..GCCT .G*G*......T C.......A. CAG
MK482687_Iran	.*G.TCG*T..G T..A..GCCT .G......GT C.G.....A. CAG

B

	1111
	112366 81223
	2379045289 31451
Sri Lanka	FWHEVIIKTR TFILD
Nepal	....
China	....
China 2	....
China 2-1	....
China 3	....
Vietnam	....
Taiwan	....
Japan	....
Russia	....
GQ379060_China	....
GU724767_India	....N..
GU724768_India	..... .L...
HM242397_India	LS..... .....
HM242399_India	...G..... .....
HM242402_India	....N
HM242408_India	....
HM242411_India	..... N....
HM242415_India	.....N.. .....
HM641257_India	.....W .....
HM641258_India	.S..... .....
KJ403739_Saudi Arabia	.....F.... .....
KJ403744_Saudi Arabia	....V.
KJ403742_Saudi Arabia	...DI.L... .....
KJ507741_Saudi Arabia	....
LN873226_Italy	.....A. .....
MK482687_Iran	.GYG..... .....

Fig. 3. Variable nucleotide (A) and amino acid sequence positions (B) of the *COI* of *Varroa destructor* haplotypes. Sequences identical to the first sequence of the Sri Lankan haplotype are indicated by a dot. Nonsynonymous nucleotide substitutions are shown in oblique and bold. Haplotypes from the parasite host of *A. cerana* are labeled in italic and those from *A. mellifera* are labeled in accession number with its acquired country.

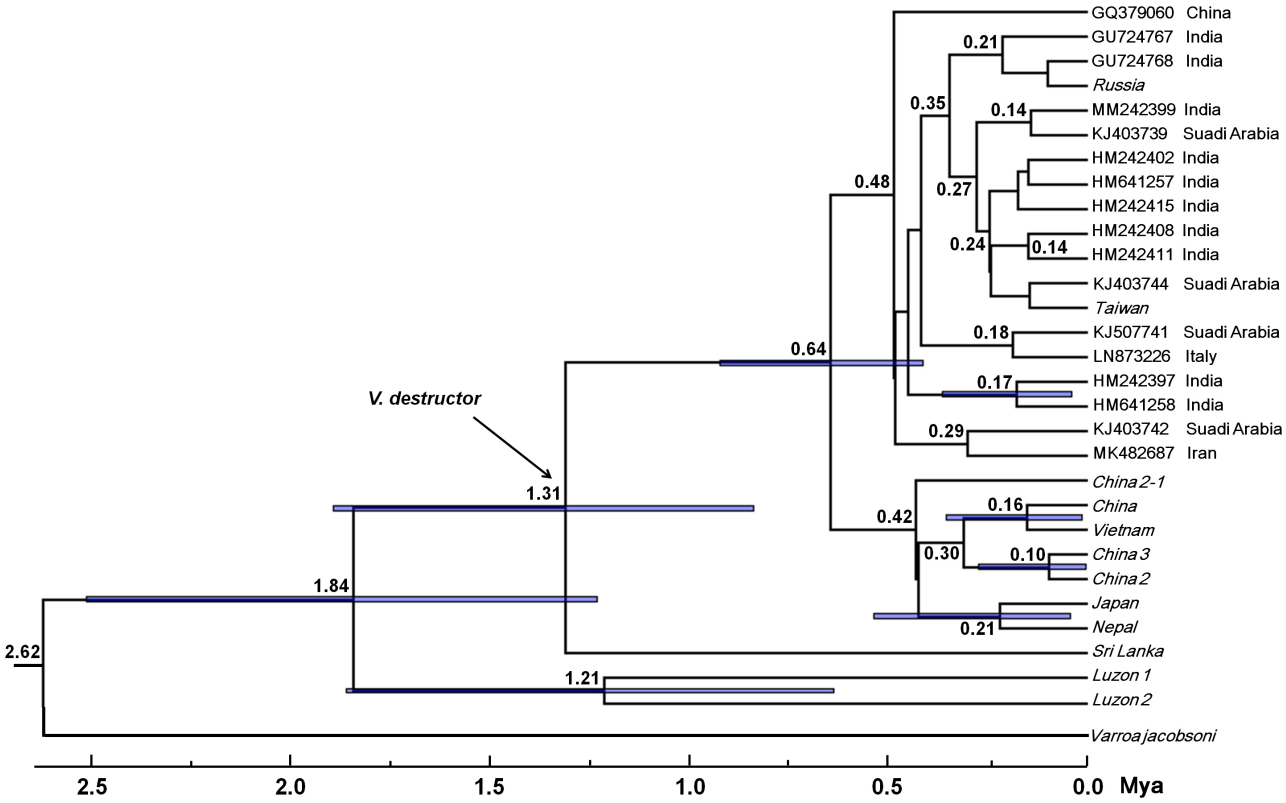

Fig. 4. Calibration dating of *Varroa destructor* based on *COI* haplotype sequences. Outgroups of *Varroa jacobsoni* and the varroan haplotypes of Luzon were used for comparisons. Divergent time is labeled in million years ago (Mya). Haplotypes from the parasite host of *A. cerana* are labeled in italic and those from *A. mellifera* are labeled in accession number with its acquired country.

of *V. jacobsoni* approximately 2.6 million years ago (Mya) and that a subsequent differentiation occurred in the Luzon and Sri Lanka lineages approximately 1.3–1.8 Mya (Fig. 4). The differentiation of the remaining haplotypes found in *A. cerana* occurred at approximately 0.10–0.64 Mya.

DISCUSSION

Morphological characteristics of *V. destructor* populations in Taiwan and other areas

Body size measurements and the target *Apis* host were found to be suitable for identifying four varroan species. *Varroa underwoodi* is the smallest in length at approximately 716 μm and is easily distinguished from the other three bigger varroan species with body lengths ranging from 1037 to 1180 μm (Delfinado-Baker and Houck 1989; de Guzman and Delfinado-Baker 1996; de Guzman and Rinderer 1999; Dietemann et al. 2013). While *V. rindereri* only parasites *Apis koschevnikovi*, its body length, approximately 1180 μm, is similar to both *V. destructor* and *V. jacobsoni*, which parasite *A. mellifera* and *A. cerana*. Moreover, a remarkably stable bimodality between body length measurements of *V. jacobsoni* and *V. destructor* was found, *i.e.*, 1037–1063 and 1106–1167 μm, respectively (Delfinado-Baker and Houck 1989; Anderson and Truemen 2000). Furthermore, the body length of the Argentinan *V. destructor* is 1178 μm (Maggi et al. 2012). In this study, the body lengths of *V. destructor* collected from *A. mellifera* and *A. cerana* in Taiwan were 1201 and 1288 μm, respectively. These sizes are slightly larger than those reported previously. When varroan specimens had body lengths longer than 1106 mm, they could be recognized as *V. destructor* rather than *V. jacobsoni* (which had lengths of 1037–1063 μm), though body length was obviously variable in *V. destructor* populations.

COI sequence differentiation in *V. destructor*

In total, 27 *V. destructor* haplotypes are found worldwide, while only J- and R-related types could reproduce in *A. mellifera* and cause serious harm (Trynor et al. 2020). Type R, however, can infect more *A. mellifera* colonies and it can induce a more severe damage than type J (Delfinado-Baker 1988; de Guzman et al. 1999; Carneiro et al. 2007). Only type J has been identified in *A. mellifera* in Taiwan, and no colony collapse resulting from a type J infestation has been recorded, although apiary cleaning and pesticide spraying were necessary (Chen 2011). Moreover, a newly discovered haplotype, named type T, an intermediate between types J and R, was found in *A. cerana* in Taiwan.

Currently, the R-related type has replaced type J in certain areas, such as Brazil, Japan, Thailand, and the United States, where mixed infestation has previously been found (Carneiro et al. 2007; de Guzman et al.1999; Dietemann et al. 2019). The genetic drift arising from host-shift events and the population expansion resulting from human activity may be responsible for the consistent genetic structure of types J and R of *V. destructor* worldwide. Although type R did not exhibit polymorphism in *A. cerana*, nearly all R tip-extending haplotypes found in *A. mellifera* in southern India, Iran, and Saudi Arabia contained amino acid substitutions, which may result from either particular environmental adaptations or other host-shift events. Moreover, new host-shift events caused by pre-adaptation or opportunistic events may have occurred because a few *V. jacobsoni* can survive in *A. mellifera* (Roberts et al. 2015).

The network outgroup comparisons, host-shift events, and artificial transportation of the current 27 haplotypes provide evidence for identifying ancestral haplotypes in *V. destructor*. The major haplotype typically recognized as an ancestral haplotype in population genetic studies may not be found in *V. destructor* because the current major J and R types had occurred through recent host-shift events in modern apiculture. The intermediate haplotypes closer to the outgroup, however, could be recognized as ancestral states that evolved prior to their tip-extended haplotypes (Fig. 2B). The local haplotypes derived from the R type found in *A. mellifera*, however, may have mutated recently.

COI sequences have been commonly applied in molecular calibration related to speciation events involving temperate Palaearctic and tropical Amazonian biota (Avise 2000; Knowles 2000; Hoorn et al. 2010; Rull 2011). Figure 4 suggests that *V. destructor* differentiated from the Sri Lankan type prior to 1.3 Mya. Ancestral haplotype retention and genetic drift subsequently occurred in local populations, as observed in the intermediate haplotypes during 0.10–0.64 Mya. This estimation was consistent with that for the period 0.15 Mya on the basis of microsatellite markers by Solignac et al. (2015). Rapidly arising mutations of *V. destructor* have occurred in *A. mellifera* from India, Iran, and Saudi Arabia, which exhibit a likely pattern of varroan mites in Papua New Guinea (Roberts et al. 2015). The differentiation history of *V. destructor* on its natural parasite host *A. cerana* was explored. The population structure inferred from morphometric and DNA sequence analyses indicated that *A. cerana*

comprises two major clusters and several submajor clusters relevant to refugia formation, with these clusters affected by Pleistocene glacial cycles (Smith and Hagen 1996; Radloff et al. 2010). Ancestral haplotype differentiation and retention in local *V. destructor* populations might be relevant to the refugium of its host during Pleistocene glacial cycles.

Hybridization in *Varroa* mites

Two independent host-shift events occurred in both the Russian Far East and Japan. The possible occurrence of R–J hybrids has been proposed because they have been observed to be sympatric in Brazil, Japan, Thailand, and the United States (de Guzman et al. 1999; Carneiro et al. 2007; Dietemann et al. 2019). A worldwide sampling analysis based on microsatellite markers revealed the occurrence of hybrid events and did not point to the presence of asymmetrical introgression between the J and R types, although many hybrids involving J alleles in the genetic background of the R type have been reported (Solignac et al. 2005; Dietemann et al. 2019). A postzygotic barrier has been proposed to serve as a mechanism for selection against type J, possibly explaining why type R replaced type J and coexisted with it in sympatric areas (Solignac et al. 2005). Examining the genetic content of R–J hybrids in sympatric areas would provide additional information on the selection fitness of the highly virulent type R relative to the less virulent type J.

Gene expression levels are key factors that can help elucidate why type R but not type J is highly virulent for *A. mellifera*. Allozyme patterns used to examine genetic differentiation furnished no recognizable differences for populations of *V. destructor* in Brazil, China and Europe (Issa 1989; Biasiolo 1992). Zhang et al. (2010) reported that *V. destructor* exhibited differences in expression of genes related to metabolic functions and nerve transmission signature of *A. mellifera* versus *A. cerana*. Oldroyd (1999) indicated that bees have been exposed to type R for the longest period in eastern Russia; thus, tolerance to *Varroa* is most common in that region, although many naturally resistant populations of *A. mellifera* have been found (Locke 2016). Moreover, on the basis of genome-wide analyses, Techer et al. (2019) found that divergent selection regimes existed between *V. destructor* and *V. jacobsoni*. In this study, network analysis indicated that type T was linked to the most common types R and J. The mechanism underlying the tolerance or virulence difference between the R and J types in *A. mellifera* could be elucidated using the available genomic sequences of the J, R, and T types.

Hybridization events between *V. jacobsoni* and *V. destructor* might be also possible. Spillbacks of *V. destructor* to *A. cerana* and spillovers of *V. jacobsoni* to *A. mellifera* have been found in sympatric populations in Thailand, where reciprocal genetic admixture has occurred between *V. destructor* and *V. jacobsoni* (Dietemann et al. 2019). The ITS sequence (EF025470) of *V. jacobsoni* was obtained from Java, Indonesia, where coinfestation of the two mites has commonly occurred (Anderson and Morgan 2007; Anderson and Fuchs 1998). Therefore, the identical ITS sequence in *V. jacobsoni* and *V. destructor* may have resulted from hybridization events. However, the fact that *V. destructor* and *V. jacobsoni* have identical ITS patterns may also have resulted from the ancestral retention of an ITS region.

Quarantine management for colonies of *A. mellifera*

Identifying the isolates of *V. destructor* species, especially those of the highly virulent type R, is crucial in the prevention of invasions of *Varroa* mites. Solignac et al. (2005) and Navajas et al. (2010) examined microsatellite markers and *COI* sequences in 12 individuals collected from Taiwanese *A. mellifera* and found pure J and J-like types of the Taiwanese *V. destructor*. In this study, a sufficient sample obtained across Taiwan revealed the presence of only the less virulent J type in *A. mellifera*. R-free regions have been recorded in some areas. Honey bee movement in each country should be examined, and quarantine requirements should be implemented for the trade of live honey bees worldwide to prevent the introduction of the R type to R-free countries, islands, or areas (Iwasaki et al. 2015; Traynor et al. 2020; Hall et al. 2021). The haplotype composition and distribution areas shown in the table S2 and figure S2 can support the clarification of existing haplotypes and examination of new haplotypes in *V. destructor*. For varroan mite management, Oldroyd (1999) also suggested that the virulent R type could be controlled by deliberately releasing the less virulent J type.

CONCLUSIONS

Two hundred and eleven *COI* were sequenced and compared with the 400 available *COI* sequences of *V. destructor*. A total of 27 haplotypes were discovered worldwide, and only the J and R-derived types were found to be able to reproduce in *A. mellifera*. In Taiwanese apiaries, the less virulent type J was found, highlighting Taiwan's success in preventing an invasion of the highly virulent type R. Honey bee

movement should be monitored worldwide to prevent the introduction of type R to areas where this type is absent. Many nonsynonymous substitutions derived from type R identified in tropical India, Iran, and Saudi Arabia might exhibit the environmental adaptation of the bee parasitic mites. Identical ITS sequences shown a possible hybridization event between *V. jabsoni* and *V. destructor* was also documented. Moreover, the newly found type T in populations of *A. cerana* in Taiwan is intermediate between types J and R, offering a possibility for studying the mechanism of virulence transformation between these two haplotypes.

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Supplementary materials

Fig. S1. Collection localities of *Varroa destructor* from apiaries in Taiwan Island. All mites were collected from host *Apis mellifera* except the Xinshe samples parasites in *Apis cerana*. The Central Mountain Range is shown, with the light gray representing the altitude between 1,000 m and 2000 m. (download)

Fig. S2. The *COI* sequences of *Varroa destructor* analyzed in this study. Pertinent information for each sequence was shown including the description with accession number (or relevant extending accession number, *i.e.*, sequence identical to the accession number defined by authors), haplotype name (or individual's abbreviation), the relevant parasite host (Acer: *Apis cerana*; Amel: *Apis mellifera*), acquired locality, and the cited reference. Pertinent information and order of sequences were also shown in table S2. (download)

Fig. S3. Bayesian phylogenetic inference for *COI* sequences of *Varroa destructor* haplotypes using outgroups of *Varroa jacobsoni* and the Luzon varroans. Posterior possibilities are shown beneath the nodes. Haplotypes from the parasite host of *A. cerana* are labeled in italic and those from *A. mellifera* are labeled in accession number with its acquired country. (download)

Table S1. Pertinent collection information of *Varroa destructor* from Taiwan, Thailand, and China. (download)

Table S2. Haplotype information of *COI* sequences of *Varroa destructor* analyzed in this study, including haplotype abbreviation and their relevant sequences number, haplotype name, accession number, acquired country with its individuals, parasite host, and the cited references. (download)