Genomic analyses of sibling honey bee ectoparasitic mite species show divergent strategies of adaptation

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Abstract

Multispecies host-parasite evolution is common, but how parasites evolve after speciating remains poorly understood. On one hand, their shared evolutionary history and physiology may propel them along similar evolutionary trajectories. Alternatively, they may pursue different strategies to reduce competition with each other. Here, we test these scenarios using the economically important association between honey bees and ectoparasitic mites by sequencing the genomes of the sister species *Varroa destructor* and *Varroa jacobsoni*. We also compare them to another honey bee mite (*Tropilaelaps mercedesae*). We find different sets of genes and gene ontology terms under selection in each of the lineages, indicating distinct selective regimes operating on each of the parasites. Divergent strategies pursued by the parasites may make it harder for the host species to develop tolerance to all of them at the same time. Based on our findings, we suggest that species-specific strategies may be needed to combat evolving parasite communities.

Interactions between hosts and parasites shape biodiversity by driving coevolutionary arms races and by regulating populations over ecological timescales. Parasitism is a remarkably successful strategy, occurring in nearly half of recognized animal phyla [1,2]. Parasites depend on their hosts, which form their principal ecological niches and evolve rapidly, often with multiple parasite generations for each host generation. This results in convergent selective regimes, leading to reproducible evolution of genomic features such as reduced genomic compaction, functional losses often associated with a reduced metabolism, and adaptive functional gains [3]. Most models of parasite coevolution are limited to interactions between single focal parasites and their hosts, although multi-species dynamics are known to be much more complex [4,5]. Furthermore, in nature, communities of related species may parasitize individual hosts [6–8]. As a result, coevolutionary dynamics involving more than one parasitic species remain poorly understood [9,10].

For example, co-infecting parasites occupying the same host (ecological niche) can exclude competitors via direct, or interference competition [11,12]. Spatial separation within regions of the host gut is a strategy to reduce interspecies competition adopted by cestodes naturally co-infecting sticklebacks [13]. Within the same host, congeneric parasites can co-exist, but spatial segregation or slight differences in niche parameters may explain intrahost speciation, as found in gill parasites (*Dactylogyrus*) of freshwater fish (*Cyprinidae*) [14]. Niche partitioning across seasons allows two fungal sibling species (*Erysiphe quercicola* and *E. alphitoides*) to exploit one host tree (*Quercus robur*) by reducing direct interferences [15]. Alternatively, parasites may shift their strategies to exploit different aspects of the host's niche. For example, fire ants (*Solenopsis spp.*) are parasitized by a genus of decapitating phorid flies (*Pseudacteon*), which are highly host-specific and have the same life cycle, but specialize on attacking ants in different contexts (*e.g.*, while foraging, *vs.* at the nest.) [16]. However, almost nothing is known about the evolutionary trajectories of speciating parasites. On one hand, they share

physiology and host-specific adaptations, which may predispose them to evolve along common lines, particularly in allopatry. On the other hand, selective regimes may be less predictable, or even disruptive, in the case of character displacement.

Here, we investigated the evolution of speciating parasites by sequencing genomes of two economically important mite species that specialize on honey bees (*Apis sp.*): *Varroa jacobsoni* and *Varroa destructor*. The honey bee colony, which is a densely packed community of genetically similar individuals, can host many diseases and parasites. Brood parasitism by ectoparasitic mites has evolved in all *Apis* species except for the western honey bee (*A. mellifera*), the only lineage non-native to Asia and allopatric with the others [17]. The two *Varroa* sister species are morphologically very similar, and until recently were thought to be the same species [18] (Figure 1A). They originally parasitized the same host, the widespread eastern honey bee (*Apis cerana*) (Figure 1B). However, as western honey bees were brought into contact with the eastern honey bee in Asia and Oceania, both mites switched repeatedly to this novel host [19,20] (Figure 1C). Since switching hosts to *A. mellifera* at the turn of the 20th century, *V. destructor* has spread worldwide, becoming a major driver of global honey bee declines [21,22]. *V. jacobsoni* evolved an ability to parasitize *A. mellifera* in the past two decades [20,23], but its potential for worldwide spread is not yet known.

While both mite species represent potential or actual threats to the survival of *A. mellifera*, they are tolerated by *A. cerana*. Both the mites and the original bee host *A. cerana* have a range of extensively studied adaptations and counter-adaptations [24–29]. In response to *Varroa*, the eastern honey bee has strategies to tolerate the parasite and to limit its propagation [25]. Specifically, the eastern honey bee has impressive host defence traits, such as worker brood incompatibility [26,27], higher toxicity response to *Varroa* saliva protein than *A. mellifera* [28,30] and hygienic behavior [31], which stop the mite reproductive cycle and reduce the infection in the brood [25]. Western honey been lack these defensive mechanisms, and hives collapse quickly after infestation by *Varroa*.

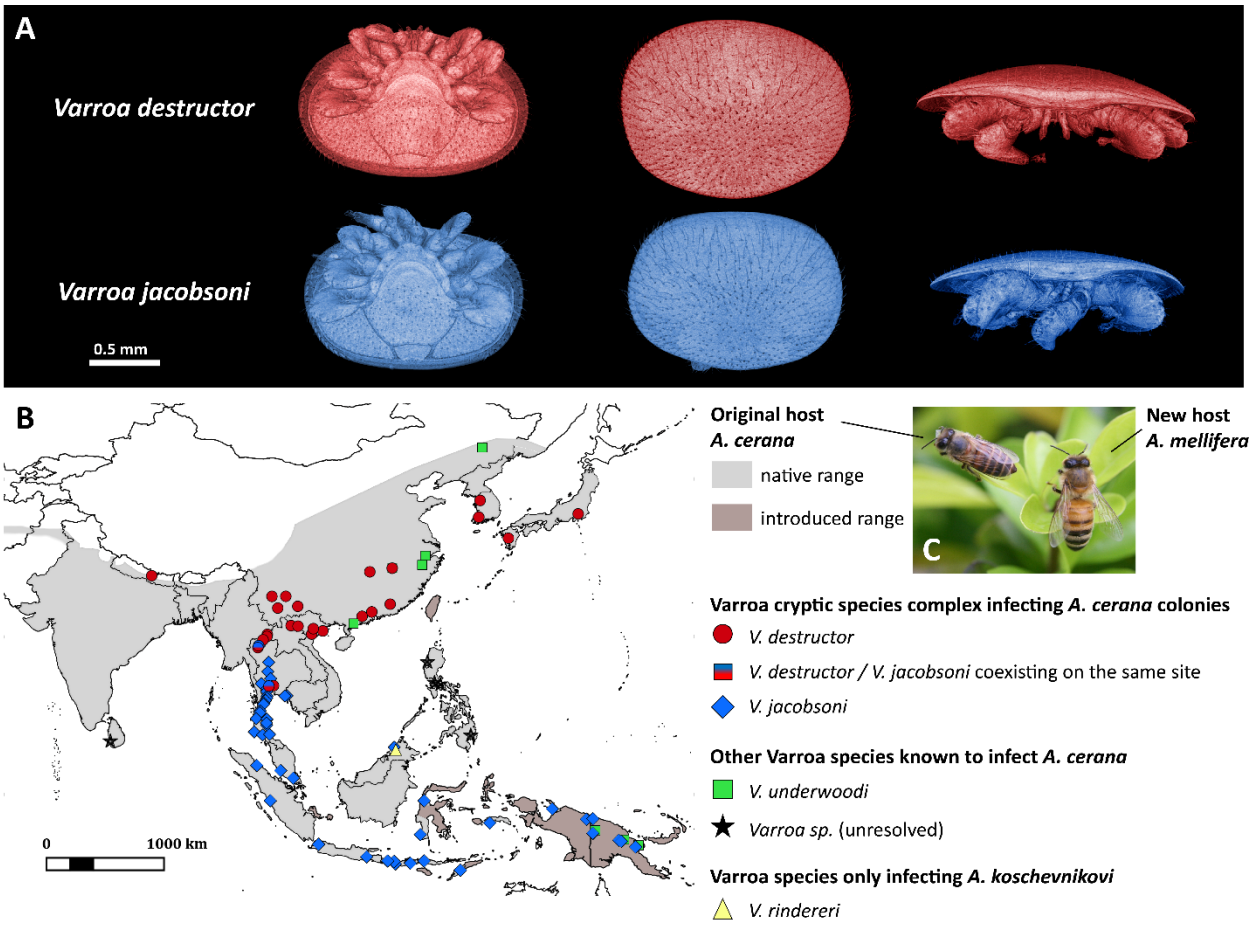

Figure 1: V. *destructor* and V. *jacobsoni* are morphologically similar sister species originally parasitizing the Asian honey bee (A. *cerana*). These species were recognized by Anderson and Trueman 2000 by quantitative morphometric and genetic data. The morphological differences are slight and rely mostly on body size, as shown on the 3D surface model comparison of two fully sclerotized females (A). Both species parasitize A. *cerana* as their original host and can coexist in sympatry, including at the same apiary [42,46], but are mainly parapatric [47] (B). With the introduction of A. *mellifera*, V. *destructor* can be found on this novel host throughout the original V. *jacobsoni* range. V. *jacobsoni* has also extended its range into Papua New Guinea on A. *cerana* followed by a shift to A. *mellifera*, where V. *destructor* is currently absent [20] (C).

On the part of V. *destructor*, which has been most extensively studied as a result of its devastating agricultural effects, behavioral and chemical ecology studies have unraveled highly adaptive strategies to find, select, feed and reproduce on its host [32]. For example, gene expression changes in the chemosensory organs on the female mite legs with the need to find a suitable host within the host colony and detect bee pheromones [33]. Mites also use chemical perception to identify the exact short time window of synchronized reproduction with the host larval development in a protected capped cell [32]. Another crucial part in V*arroa* parasitic lifestyle is the ability to feed on its host to respond to energy demand linked to egg production and survival [34]. The behavioral and metabolic processes involved have recently been majorly updated as mites assumed hematophagous were shown feeding on fat tissues with the detection of host fat body proteins and transcripts in V*arroa* [35,36], microscopic observation [37], and chemical analysis of parasite waste excretion [38]. While many mite adaptations have already been discovered [32,39], the recent use of 'omics approaches has quickly broadened our understanding of the mite's hidden specialization to host [29,36,40,41].

Here, we ask how both mite species have arrived at this equilibrium in A. *cerana* – did they follow similar or different evolutionary paths? On one hand, the two species naturally occur in parapatry (Figure 1B), which is expected to result from divergent local adaptation with the distribution of different A. *cerana* lineages [42,43]. In actuality, what drives the parapatric distribution is unclear. V. *destructor*, which was historically absent from Southeast Asia, now occurs there on its novel host A. *mellifera* in sympatry with V. *jacobsoni*, which does not parasitize A. *mellifera* in this region (Figure 1C). Thus, it is possible that the current geographical distribution resulted from secondary contact following allopatric speciation as shown in other closely related species like the alpine rock-jasmine [44]; and similar evolutionary trajectories and competitive geographic exclusion like in the case of chipmunks (E*utamias dorsalis* and E. *umbrinus*) [45]. This view is further supported by the fact that both species have the genetic capacity to shift to A. *mellifera* as a novel

host, suggesting a level of physiological similarity. We tested these alternative hypotheses (parallel *vs.* divergent evolution) by generating high-quality genomes of both species and examining them for signatures of adaptive evolution. We also compared them to the recently published genome of a distantly related ectoparasitic mite *Tropilaelaps mercedesae*, to see whether the patterns appear universal at different timescales.

Materials and Methods

V*arroa* mite sample collection

V. *destructor* and V. *jacobsoni* are haplodiploids. Haploid males are only found inside capped brood cells after initiation of reproduction while diploid females can be found in the brood and on adult workers. All V. *destructor* samples were collected from A. *mellifera* colonies of OIST experimental apiary in Okinawa, Japan (26.477 N 127.835 E) in August-September 2016. One mature male V. *destructor* was collected within a worker cell at the red-eye pupa developmental stage using a brush and kept in absolute ethanol at -80°C. Mature adult females were collected from adult honey bee workers. In order to obtain a large number of mites infecting adult workers, we modified the standard powdered sugar method [48] for an entire hive as follows: one hive super was placed on top of a white collecting tray, containing three to four frames with honey bee workers and no queen; approximately 500g of powdered sugar was then applied using sieve filter powder strainer; the sugar and honey bees were shaken in the tray for 2 min and then separated using a sieve filter, placing the sugared-workers directly within hive. This process was repeated on adjacent colonies. V*arroa* mites were separated from their hosts and trapped in the icing sugar. Following a water rinse on a gauze mesh, a total of 1,207 alive V*arroa* females were snap frozen at -80°C until laboratory processing (inactive, sluggish or dead mites were discarded). One fully sclerotized mature female V. *destructor* (VDOKI-01) collected from the same apiary in reproductive phase was processed for X-ray microtomography (micro-CT). Micro-

CT outputs were focused on the *Varroa* idiosoma ventral and dorsal views, and whole body in profile to show the mite anterior and gnathosoma parts.

Samples of *V. jacobsoni* were obtained from its first detection survey where it was found reproducing in the western honey bee [20]. One mature male (VJ856) collected from *A. mellifera* drone brood cell in Papua New Guinea (between EHP and Henganofi border, 30/05/2008) was used to prepare whole genome library. One fully sclerotized mature female *V. jacobsoni* (VJ347-11) collected from *A. cerana* originating in Java (Cililin central Java, 13/06/1998 by D. Anderson) was processed for micro-CT.

DNA extraction, genome sequencing, assembly, and annotation

Genomic DNA was extracted from each whole haploid male *V. jacobsoni* and *V. destructor* using QIAamp DNA Micro Kit (© Qiagen) following manufacturer's instructions. The total amount of dsDNA was measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen). Short-insert of 250 bp paired-end libraries were prepared for both individuals using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, Inc) and 10 PCR cycles. Size-selection and cleanup were applied using SPRI beads. Each library was then checked using a Bioanalyzer High Sensitivity DNA kit (Agilent). Both paired-end libraries were each sequenced on two lanes of HiSeq 2500 (Illumina ®) at the Okinawa Institute of Science and Technology (OIST) sequencing center in paired-end 250 cycle mode.

Prior to assembly paired-end reads were de-duplicated using ParDRe [49], and host and microbial contamination was filtered using DeConSeq (parameters: -i 97 -c 95) [50], screening against genomes of *Apis cerana*, *Apis mellifera*, as well as bacterial genomes and genomes of bee diseases and parasites. The cleaned reads were then merged using PEAR (parameters: --min-overlap 10 --memory 80G --threads 10 -n 200 -m 600 -p 0.0001) [51] and assembled using Newbler (v. 2.6) (parameters: -large -m -cpu 24 -mi 98 -siom 390 -l 1000 -a 500 -urt -novs -a 1000 -minlen 45 -ml 100) [52]. The contigs were then scaffolded using publicly available

RNA-seq data assembled using Trinity [53,54]. For V. *destructor* further scaffolding was carried out using Hi-C, performed by Phase Genomics. Hi-C read pairs were aligned to the initial assembly using BWA-MEM with the "-5" option [55], keeping only primary properly paired alignments (samtools with the "-F 2316" filter [56]). Scaffolding was then performed using Phase Genomics' Proximo Hi-C scaffolding pipeline [57], which builds on the LACHESIS method [58].

To estimate genome characteristics, K-mers from cleaned reads were counted using jellyfish version 2.2.7 [59] for the following values of K: 19, 25, 31 and 42 and coverage cut-off of 1,000. Frequencies were only computed on HiSeq 2500 reads from the individual haploid male of each species. Computed and selected K-mer histograms for k=42 were analyzed with GenomeScope to estimate genome size, kcoverage, number of duplicates [60]. We used the bioinformatically derived genome sizes for genome coverage estimation.

Small batches of adult females V. *destructor* (n = 1,207) were pooled and crushed by pestle in 1.5 mL Eppendorf tubes. The powder was then lysed using proteinase K and lysis buffer at 56℃ for 20 min. Sample lysate was thus transferred to a Maxwell Cartridge. The remaining step was fully performed on the Maxwell 16 automated purification system (Promega) with the LEV Blood DNA mode. Library preparation for PacBio RSII sequencing was performed using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) following manufacturer's recommendations. SMRTbell libraries were prepared and sequenced at the OIST sequencing center on 48 SMRT cells. These reads were trimmed using proovread [61] and then used to gap-fill the Hi-C scaffolded V. *destructor* assembly using PBJelly (parameters: -minMatch 8 -minPctIdentity 70 -bestn 1 -nCandidates 20 -maxScore -500 -nproc 24 -noSplitSubreads) (v. 15.8.24) [62].

Annotation for both genomes was carried out by NCBI using NCBI's automated genome annotation pipeline. This pipeline takes advantage of species-specific RNA-seq data, as well as extensive protein homology data stored in GenBank [63].

Prediction of gene orthology, selection, and duplication in V*arroa* mites

The Orthonome pipeline (http://www.orthonome.com) [64] was applied to
235 predict orthologues (pairwise and 1:1 orthogroups), inparalogues and lineage-specific gene expansions from the two parasitic V*arroa* mites' annotated genomes and four Acari annotated genomes available from NCBI. The four outgroups were the Asian honey bee ectoparasitic mite *Tropilaelaps mercedesae* [65], the parasitic tick *Ixodes scapularis* [66]; the free-living Western predatory mite *Metaseiulus occidentalis*
240 [67] and the free-living two spotted mites *Tetranychus urticae*. Total and pairwise/lineage intersection of orthologs gene sets were represented using Upset plots [68] using R. The 1:1 orthologues with both V*arroa* species represented were used for positive selection hypothesis tested on phylogeny using HyPhy [69]. Additionally, tandem duplications in the three parasitic mites were identified from
245 inparalogues in the immediate vicinity of the orthologues.

Gene ontology (GO) enrichment analysis of the positively selected genes in honey bee parasitic mites was carried out using the GOstats R package [70]. The same process was applied for gene duplication in V*arroa* mites. Biological processes associated with those GO terms were summarized and visualized using REVIGO [71]
250 (http://revigo.irb.hr). The semantic similarity threshold used was C = 0.5 (i.e., small). The resulting source code generated online was then used to produce a scatter plot and treemap plot using the ggplot2 package. The same package was used to plot the localization of the genes under positive selection and duplicated onto V. *destructor* chromosomal scaffolds.

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Identification of V*arroa* mite strains using mtDNA and sequence similarity

Most previous taxonomic work relied on mitochondrial fragments. We used mitochondrial analysis to determine and compare the lineages of V*arroa* species used for genome sequencing to the previous described haplotypes. The whole
260 mitogenome of V. *destructor* (accession NC_004454.2 [72,73]) was available on NCBI

database and used as a reference for read mapping. Raw Illumina reads for both V. *destructor*, and V. *jacobsoni* males were mapped onto the entire reference sequence (16,476 bp) using NextGenMap 0.5.0 [74]. Mapped reads were sorted, and PCR duplicates were removed using Samtools 1.3.1 [56]. Maximum coverage of 500x was subsampled on all mapped reads using VariantBam 1.4.3 [75]. Subsequently, variant calling was performed using FreeBayes v1.1.0-46 [76], with following parameters others than the default: minimum mapping quality of 20 and minimum base quality of 5. The same mapping method was used to estimate the sequence similarity between the two V*arroa* genomes, in which Vjacob_1.0 reads were mapped against the reference Vdes_3.0 to detect the number of variants. Consensus mitochondrial sequence was generated for each individual using vcf2fasta from Vcflib library [77]. A partial sequence of *Cox1* gene (426 bp) was extracted and aligned together with 26 V*arroa spp.* haplotypes from the taxonomic revision study and addition [18,78–80]. V. *destructor* lineage was further identified by comparing partial and concatenated *Cox1*, *Cox3*, *Atp6* and *cytB* alignment with 22 other sequences [78,81]. Sequences were aligned using ClustalW, checked by hand and a consensus maximum likelihood tree was constructed after 5000 bootstraps in Mega 7.0.18 [82].

A distribution map of the V*arroa* mites infecting A. *cerana* was built from reviewing literature. Conservatively, only the presence of V*arroa spp.* confirmed by mtDNA sequencing after the taxonomic revision was plotted [18,42,78–80,83–85] onto the potential native and introduced range of the Asian honey bee host [43,86]. The map was produced using QGIS 2.16.1 (Open Source Geospatial Foundation Project http://qgis.osgeo.org) and the public domain natural 10m earth raster NE1_HR_LC (https://github.com/nvkelso/natural-earth-raster).

Results

An improved *de novo* assembly at chromosomal level for V. *destructor*

We generated 28.6Gb (333 ± S.D. 56 bp) and 56.2 Gb (409 ± S.D. 61 bp) of de-duplicated, decontaminated and merged Illumina reads for V. *jacobsoni* and V. *destructor*, corresponding to a coverage of 80× and 152×, respectively.

Through the k-mer analysis the genome size estimates ranged from 369.02 to 369.55 Mbp using k = 42 (Figure S1). The overall final Vdes_3.0 assembly size was close to that estimate reaching 368.94 Mbp. The final size represented a significant improvement of 25.4% in comparison to the first release assembly Vdes_1.0 [87] and 11.2% to the later corrected into Vdes_2.0 (Table 1). Not only the overall assembly size, but the contiguity of Vdes_3.0 was highly and significantly improved compared to the previous released assemblies. According to the N50 statistics, more than 50% of the genome was in scaffolds of 58.5 Mbp or more for Vdes_3.0 while the Vdes_2.0 N50 was around 0.1 Mbp. Comparison of genomic contiguity statistics with other invertebrate assemblies revealed that Vdes_3.0 currently has the best reported scaffold N50 in arthropods and best contig N50 for Mesostigmata as reported by [88]. The new V. *destructor* genome assembly was scaffolded at chromosomal scale using Hi-C data. Seven major scaffolds were constructed ranging from 39.4 to 76.9 Mbp with additional of 1,418 nuclear minor scaffolds > 0.2 Mbp. The longest genomic scaffolds correspond to the seven haploid chromosomes (2n = 14) previously detected in V. *destructor* karyotype [89]. PacBio sequencing yielded 20.3 Gb of V. *destructor* reads with a N50 of 13.5 kb (66× coverage), which was used to fill 84.4% of the gaps in the scaffolded HiC assembly.

Table 1: Improvement and release of new genome assembly statistics for honey bee *Varroa* mites

		V. destructor			*V. jacobsoni*	*T. mercedesae*
Genome assembly		**BRL_Vdes_1.0**	**Vdes2.0**	**Vdes_3.0**	**Vjacob_1.0**	**T. mercedesae_v01**
NCBI accession		GCA_000181155.1	GCA_000181155.2	GCA_002443255.1	GCA_002532875.1	GCA_002081605.1
	Assembly size (Mb)	294.13	331.92	368.94	365.59	352.54
	Coverage	5x	60x	119x	57x	60x
	Gap size (bp)	0	2,821,918	271,335	408,908	26,412,611
	GC content (%)	-	40.1	40.9	40.9	40.9
Scaffold	Scaffolds (n)	-	20,448	1,426	4,881	33,764
	N50 scaffold length (bp)	-	128,078	58,536,683	233,810	28,859
	L50 scaffold length (bp)	-	703	3	482	3,629
Contig	Contigs (n)	184,190	52,152	4,498	8,241	83,645
	N50 contig length (bp)	2,262	15,568	201,886	96,009	12,742
	L50 contig length (bp)	40,912	6,465	556	1,168	6,899
Annotation	Gene number (n)	-	-	12,849	15,486	15,190
	Mean length (bp)	-	-	18,634	14,657	5,298
	mRNA number (n)	-	-	30,208	26,214	-
	Mean mRNA length (bp)	-	-	4,193	3,489	-
	CDS number (n)	-	-	30,208	26,214	-
	Mean CDS length (bp)	-	-	1,953	1,695	1,117
	Intron number (n)	-	-	94,154	93,749	-
	Mean intron length (bp)	-	-	3,299	2,898	788
	Exons number (n)	-	-	110,06	111,624	-
	Mean exons length (bp)	-	-	537	487	407
		from Cornman *et al.* 2010		from this study	from this study	from Dong *et al.* 2017

The first draft genome for the mite V. *jacobsoni*, a novel parasite of A. *mellifera*

For V. *jacobsoni*, the assembly was performed using whole genome shotgun sequence from a Hiseq 2500. Prediction of V. *jacobsoni* genome size by k-mer frequency was slightly lower than V. *destructor* with an estimated model of 365.13 Mbp (Figure S1). The accuracy and heterozygosity were estimated to be 99.6% and 0.06%, respectively. Similarly, for its sister species, the draft genome for V. *jacobsoni* was close to that value with an assembly size of 365.59 Mbp. In total, 8,241 contigs were assembled into 4,881 scaffolds for Vjacob_1.0 with an N50 of 233,810

bp. The GC content composed 40.9% of the assembly, same as for the sister species Vdes_3.0. Contrary to Vdes_3.0 which beneficiated of long reads and chromosome capture sequencing, Vjacob_1.0 presented twice more gap in the assembly (0.41 Mbp) but yet almost seven times less than Vdes_2.0. As in October 2017, the automated NCBI Eukaryote annotation pipeline predicted 12,849 genes for Vdes_3.0, 15,486 genes for Vjacob_1.0 [63]. When mapped onto the Vdes_3.0 reference genome, a total of filtered 1,125,230 biallelic SNPs were detected. Relative to Vdes_3.0 genome size, sequence divergence with Vjacob_1.0 elevated to 0.30% across nuclear genome sequence.

Mitochondrial identification of V*arroa* mites

Mapping and pairwise whole mitochondrial alignment (16,505 bp) showed that Vdes_3.0 diverged of only 0.1% to the reference V. *destructor* mitogenome (NC004454.2) [72,73] (Figure 2A). Despite presenting similar morphological phenotypes mainly distinguishable through female body size [18,48] (Figure 1A), V. *jacobsoni* mitogenome diverged from Vdes_3.0 mtDNA with 5.1% of nucleotide differences. Given that *Cox1* is a standard marker used to identify V*arroa* mites "haplogroup", the region was extracted and realigned with described sequences from native and introduced populations. The divergence within *Cox1* complete sequence for V. *destructor* and V. *jacobsoni* reference genome rose up to 5.9% of sequence dissimilarities. The partial *Cox1* sequence (426 bp) of Vdes_3.0 clustered with V. *destructor* sequences and was distinct from Vjacob_1.0 which clustered with V. *jacobsoni* haplotypes (Figure 2B). The concatenated *Cox1*, *Atp6*, *Cox3*, and *Cytb* sequence (2,696 bp) from Vdes_3.0 mitogenome was 100% similar to the global invasive Korean K1-1/K1-2 sequence (Figure 2C). For V. *jacobsoni* Vjacob_1.0 i) the partial *Cox1* region (426 bp) was a unique and new haplotype but 99% similar to Java (one transition point mutation G/A) and Bali (two transitions) haplotypes and ii) the concatenated sequence (only *Cox1*, *Atp6*, and *Cox3*) was also a distinct haplotype from V. *jacobsoni* Laos 1 and 2. This mtDNA haplotype of Vjacob_1.0, named here

Papua New Guinea, was already detected in field surveys and reported to have switched host on A. *mellifera* (Roberts, unpublished data).

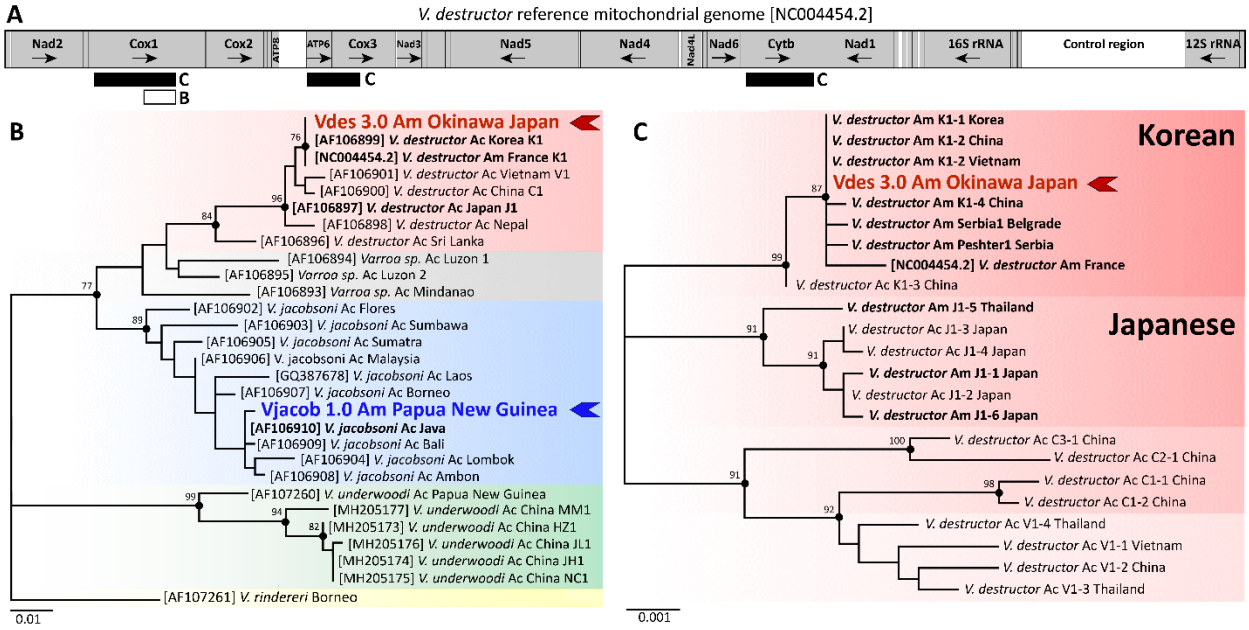

Figure 2: Inter and intraspecies mtDNA variability across the genus *Varroa* and host switched lineages. Different mitochondrial markers were developed to discriminate the four *Varroa* species (and unresolved one) based on partial *Cox1* [18] (A, B) and intraspecies lineages using larger *Cox1*, *ATP6-Cox3* and *CytB* regions [78,81] (A, C). The two cryptic species V. *destructor* (red) and V. *jacobsoni* (blue) are genetically divergent as shown by the unrooted phylogenetic tree of *Varroa* mites partial *Cox1* sequence (B). Variability within V. *jacobsoni* is higher than any other *Varroa* species, and the reference genome (blue arrow) corresponded to one of the previously detected haplotypes switching on A. *mellifera* in Papua New Guinea [20] (B). Several haplotypes from the Korean and Japanese lineages successfully jumped on A. *mellifera* (bold) [78,81], but only K1-1/K1-2 is quasi-cosmopolite and can even be retrieved in the native range of Japanese lineage as illustrated by its presence in Okinawa (red arrow) (C). Ac: identified from *Apis cerana*, Am: identified from *Apis mellifera*.

370 **Predicting orthology relationships between *Varroa* mites and other Parasitiformes mites**

The arthropod genome database at NCBI presently contains only two other Mesostigmata genomes: the non-parasitic Western predatory mite *Metaseiulus* (= *Typhlodromus or Galendromus*) *occidentalis* [67] and the honey bee parasitic mite
375 *Tropilaelaps mercedesae* [65]. Orthologue prediction was carried by comparing the annotated *Varroa* mites genes to the two other Mesostigmata species gene set and two additional Acari: i) the parasitic tick (*Ixodes scapularis*) and ii) the free-living two-spotted mite *Tetranychus urticae*. The tick presented the highest set of genes (n = 20,486) and also possessing a haploid genome more than four times bigger 1.76
380 Gb [66]. Whereas the two spotted mites showed the less complex genome regarding its size of 90.8 Mb and the lowest GC content (32.5%). Overall the two *Varroa* mites assemblies showed the best contiguity statistics, genome coverage and smaller gap size (Table 1).

Orthology prediction analysis using the six species' genomes, clustered the
385 genes by domain into 7,502 super orthologues groups consisting of orthologue and in-paralogue [64]. A total of 11,123 orthologue groups were predicted, and 42.8% were shared by the five parasitiformes and the outgroup spider mite *Tetranychus* (Supplementary Table 1). The concatenated phylogenetic tree built on the genomic sets based on 4,758 genes using IQ-Tree [90] confirmed the close relationship
390 between the three mite species compared to other Acari (Figure 3). Within the honey bee ectoparasitic mites, *Varroa* and *Tropilaelaps* shared 813 lineage-specific orthogroups (akin to gene families) for which 65.9% were Varroidae-lineage-specific. The two siblings *Varroa* shared 9,628 of orthologous genes.

The orthologous genes lineage specific to Parasitiformes were enriched for
395 molecular functions and biological processes involved in several transporters and channel activity (Supplementary Table 2). When considering the Mesostigmata orthologous sub-group (i.e., *M.occidentalis*, *T. mercedesae* and both *Varroa*), GO terms related to the structural constituent of the cuticle were the only significantly

enriched. At the *Varroa* genus level, orthologous genes showed GO terms mainly related to endonuclease activity thus implicated in the cleavage of DNA/RNA.

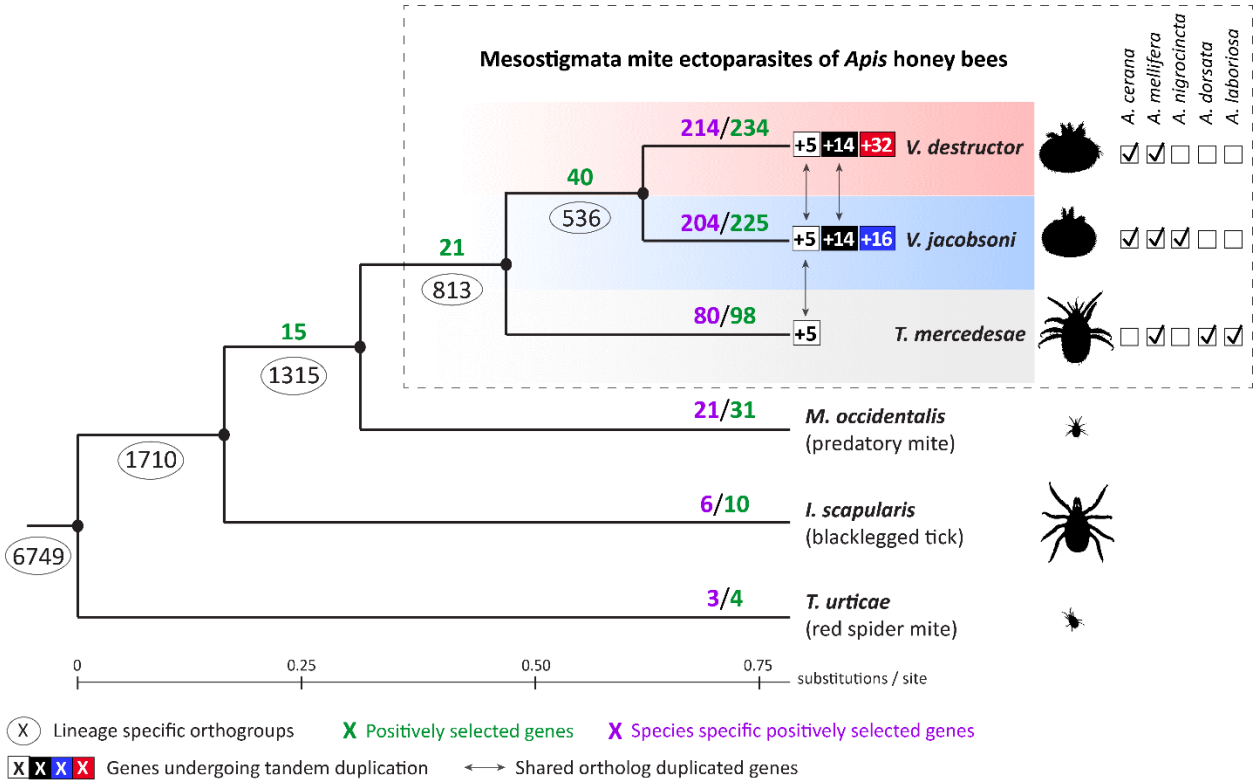

Figure 3: Positive selection and gene duplication of orthologous genes in parasitic and free-living Acari. For the honey bee mite taxa, host ranges are shown as checkboxes and had to comply the following conditions: 1) mites were found reproducing or observed within colony in independent surveys, and 2) the identity of cryptic *Tropilaelaps* and *Varroa* species was confirmed by molecular markers (e.g., *Cox1* barcoding [18,91]). The number of shared genes in each lineage is circled at the nodes. The total number of positively selected genes is shown in green, with the number of species-specific genes shown in purple. Gene duplications within honey bee parasitic mites are shown by boxes: shared genes by white squares, *Varroa*-specific genes by black squares, and lineage-specific genes by red and blue squares. The two *Varroa* mites have similar host ranges, through *V. jacobsoni* also occurs on *A. nigrocincta*, a close relative of *A. cerana*, possibly as a result of a recent secondary host shift [18,48]. Despite sharing the same ancestral host, the *Varroa* sister species show different patterns of adaptive molecular evolution and exhibit differential gene expansion, suggesting different evolutionary trajectories.

Divergent patterns of selection and gene expansion among three honey bee ectoparasitic mites

420 We detected a total of 234 and 225 genes under positive selection for V. *destructor* and V. *jacobsoni*, respectively (Figure 3). These gene sets were largely non-overlapping, with 91.4% and 90.6% of the genes under positive selection being species specific for V. *destructor* and V. *jacobsoni*, respectively (Figure S2, Supplementary table S2-S5). The remaining proportion was mostly positively
425 selected genes shared by the three honey bee mites rather than any other Acari which represented 6.8% of the positively selected genes in V. *destructor* and 7.1% in V. *jacobsoni*. The genomic regions under positive selection were distributed all along each major chromosome of V. *destructor*, and, assuming preservation of synteny, in V. *jacobsoni* as well (Figure 4A). Other than the genes under positive
430 selection at the Varroidae ancestral lineage (n = 40) and common to the two sister species (n= 12), locations on the chromosomes involved differed between V. *destructor* and V. *jacobsoni*. The differences persist at the functional levels shown by GO terms. Semantic distribution of the GO terms for the genes under selection in each V*arroa* species showed no overlap in the biological processes, suggesting
435 functionally different routes to host adaptation (Figure 4B, Supplementary Table S7). In V. *destructor*, GO terms were mainly associated with 1) the regulation of membrane depolarization, 2) retinal cone cell development and myofibroblast differentiation, 3) mRNA cis splicing via spliceosome, 4) cellular response to alcohol (i.e. any process that results in a change or activity of a cell) and more detailed in
440 Figure S3A. In V. *jacobsoni*, GO terms related to 1) protein processing involved in targeting to mitochondrion, 2) vitamin K metabolic process, 3) response to pH, 4) gonad development l and more processes detailed in Figure S3B.

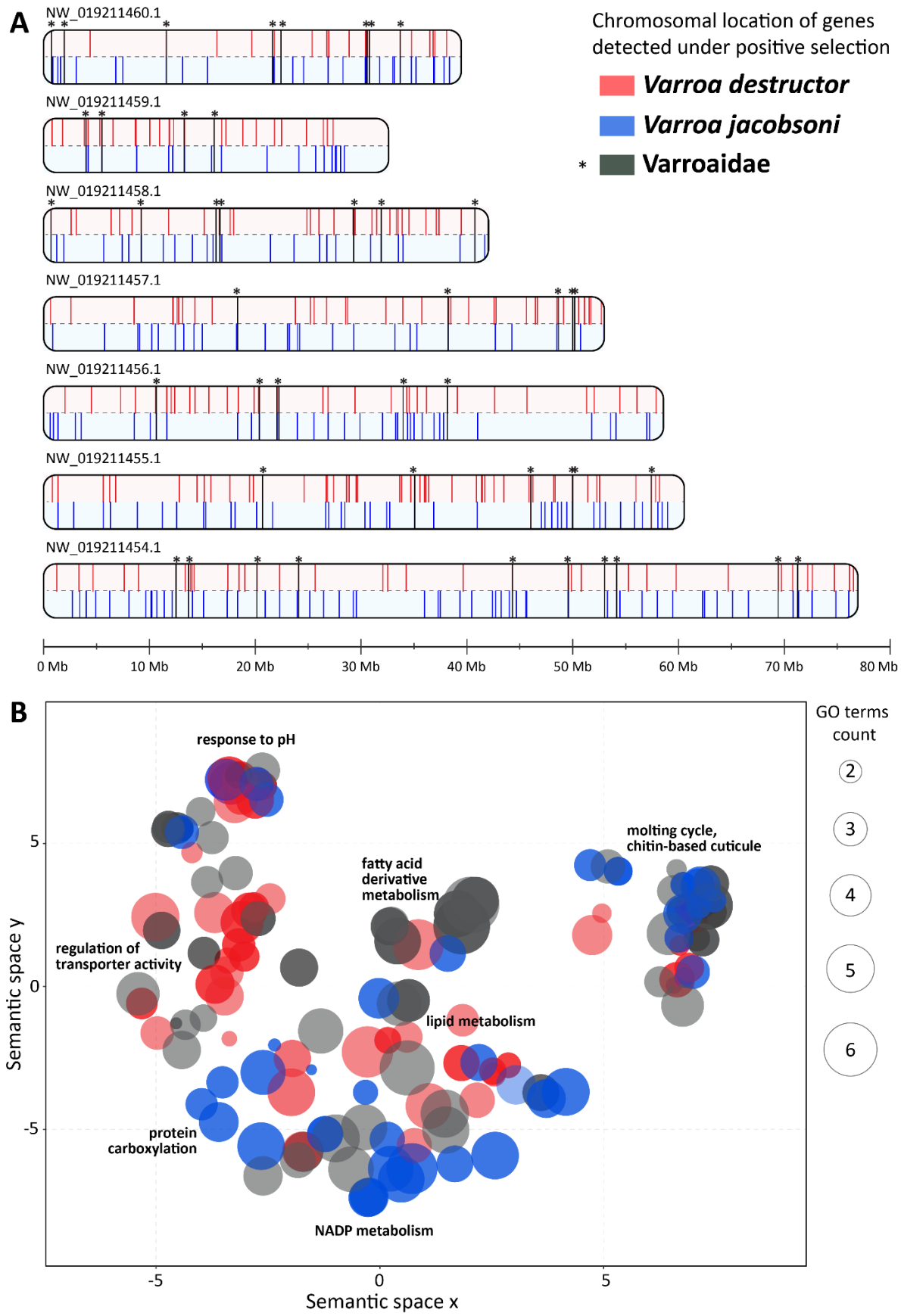
A
NW_019211460.1
NW_019211459.1
NW_019211458.1
NW_019211457.1
NW_019211456.1
NW_019211455.1
NW_019211454.1
Chromosomal location of genes detected under positive selection
Varroa destructor
Varroa jacobsoni
Varroaidae
0 Mb
10 Mb
20 Mb
30 Mb
40 Mb
50 Mb
60 Mb
70 Mb
80 Mb
B
response to pH
molting cycle, chitin-based cuticule
fatty acid derivative metabolism
regulation of transporter activity
lipid metabolism
protein carboxylation
NADP metabolism
Semantic space y
Semantic space x
GO terms count
2
3
4
5
6

Figure 4: Different genes and pathways under positive selection in the V*arroa* sister species. Red and blue bars (in 5 kb windows) represent locations of genes in V. *destructor* and V. *jacobsoni*, respectively (A). The V. *jacobsoni* data are mapped to the V. *destructor* scaffold positions for comparison purposes. The black asterisks indicated genes under positive selection, shared by both species (n = 12) or detected in V*arroa* ancestral lineage (n = 40). Semantic space analysis of the significantly enriched GO terms for biological processes over-represented among genes detected under positive selection for V*arroa* mites (B). Bubble color indicated for which species GO terms were enriched (all p-value < 0.05) and size indicates the frequency of the GO term found in the GOA database. There was little overlap in analyses at both gene and functional levels, suggesting different selective pressures on the two sister species since they split.

Tandem duplication was detected in 51 genes for V. *destructor*, 35 in V. *jacobsoni* and 70 in T. *mercedesae* (Figure 3). The number of duplication events within a species and gene cluster varied from two to six units and generally involved adjacent chromosomal locations in V. *destructor* (Figure 5A, Supplementary Table S7). Only five duplicated gene clusters were shared by all three honey bee parasite species that is 9.8% and 14.3% of detected tandem duplication for V. *destructor* and V. *jacobsoni*, respectively (Figure 2, Supplementary Table S8). GO terms associated to these honey bee mite gene duplicated clusters related to several metabolic processes including chitin, amino sugar, drug and carbohydrate derivative. Conversely, the two V*arroa* sister species shared 14 gene clusters undergoing tandem duplication, representing 27.5% and 40.0% of the total size for V. *destructor* and V. *jacobsoni*, respectively. When summarizing the list of shared duplicated genes by V*arroa*, GO terms were associated to biological processes in glycerophospholipid catabolism, body development, regulation of DNA-templated transcription/elongation and amino-sugar metabolism (Supplementary Table S9). Finally, 62.7% of the duplicated genes were exclusive to V. *destructor* whereas 45.7% were only detected in V. *jacobsoni*. On one hand, genes duplicated in V. *destructor* were associated with GO terms related with biological

processes such as mechanical/external stimulus, regulation of endoribonuclease activity and phagocytosis (Figure 5B, Supplementary Table S10). On the other hand, genes duplicated in V. *jacobsoni* were involved in biological processes of the skeletal myofibril assembly, Golgi calcium ion transport, striated muscle contraction (Figure 5B, Supplementary Table S11).

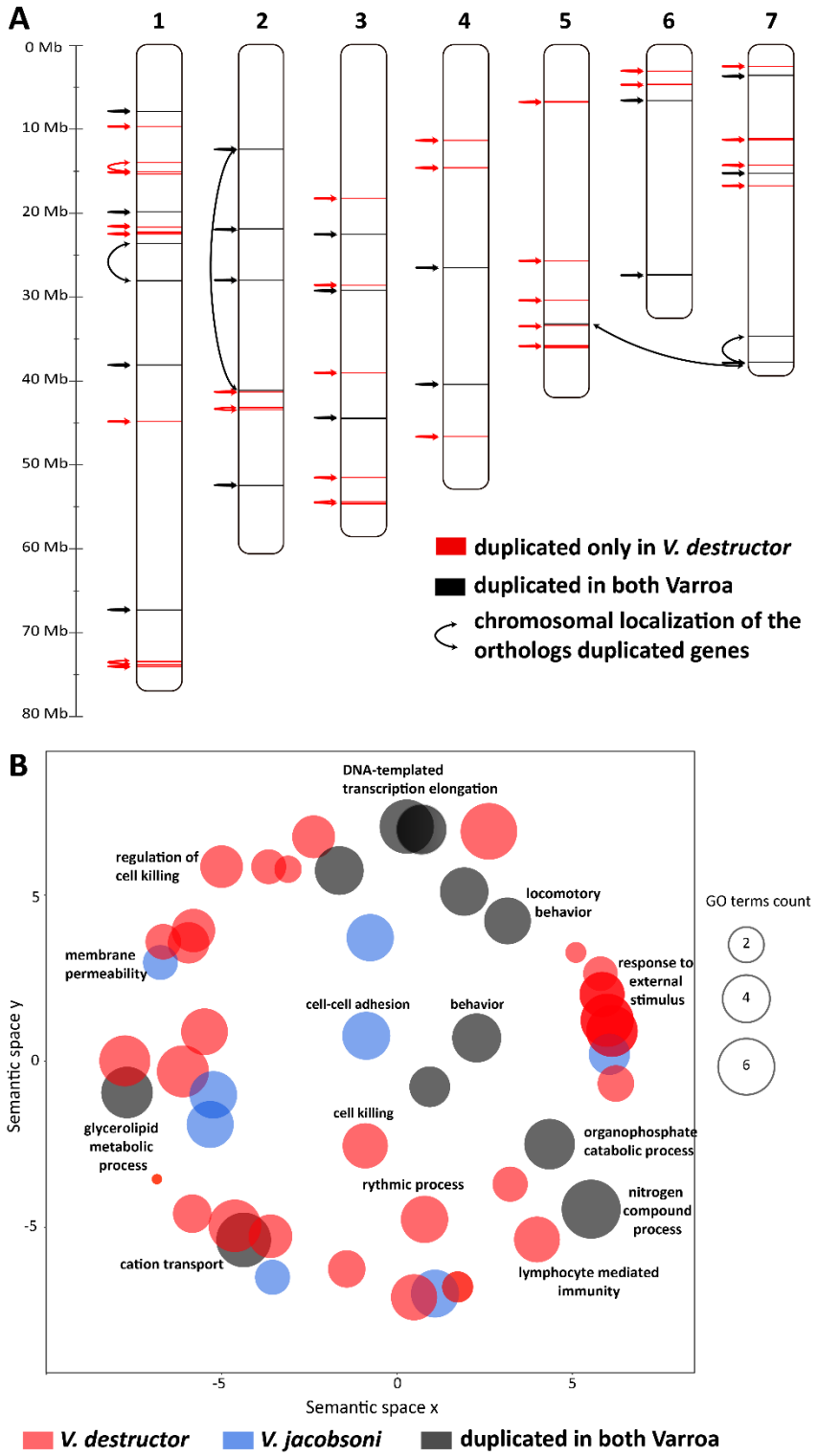

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Figure 5: Duplicated genes in V. *destructor* are found all along the genome and are involved in different biological pathways. (A) Chromosomal location and similarity of the

duplicated genes (arrows) for V. *destructor*. The different genes and chromosomal regions underwent duplication in the two species. (B) Cluster analysis of the significantly enriched GO terms for biological processes over-represented among duplicating genes in V*arroa* mites. Not only were most of the GO terms species-specific, but they were also responsible for non-overlapping categories of biological processes. Neither gene duplication nor selection analysis suggests substantial degrees of parallel evolution in the mites.

Comparative genomics of detoxification systems and other metabolic pathways highlight different selective trajectories between V. *destructor* and V. *jacobsoni*

Some annotated genes detected under positive selection or undergoing tandem duplication in V*arroa* has been previously linked to play a role in external stress tolerance (e.g., in-hive temperature environment), nutrition, molting, reproduction and metabolizing toxic xenobiotics possibly leading to acaricide resistance (detailed in Table 2). Interestingly, both species have genes under positive selection that are known to be involved in acaricide resistance. These include 12 genes such as esterases involved in detoxification, and possible changes in transmembrane proteins and channels, which were found in V. *destructor*. Nine genes with similar functions were under positive selection in V. *jacobsoni*. In V. *destructor* chromosome 4 (NW_019211457.1), two duplicated genes were also under positive selection and were involved in adult-specific rigid cuticular proteins. These genes are involved in the physical properties of the mite cuticle thickening, which in addition to providing protection may constitute the first barrier to reduce the penetration of external chemical agents [92]. On the other hand, two duplicate clusters were also detected undergoing positive selection and annotated as part of the ABC family (ATP-Binding Cassette sub-family A) and to aminopeptidase M1-B-like, in V. *jacobsoni*. Finally, the most remarkable tandem duplication occurred on Vdes_3.0 chromosome 1 (NW_019211454.1) with six duplicated genes coding for heat shock 70 kDa protein whereas none was detected for V. *jacobsoni* (Table 2). Both positive selection and duplication patterns along V. *destructor* and V. *jacobsoni*

genomes and related GO terms showed that the two sister species underwent dissimilar evolutionary trajectories.

Table 2: Genes undergoing positive selection or duplication, which were previously implicated in tolerance to external stress sources and stimuli. Previous research on Acari and V. *destructor* has identified a large number of genes involved in stress and, detoxification that can lead to acaricide resistance. Intriguingly, many of the genes appear to be under positive ancestral selection, as (a) V. *jacobsoni* has not been extensively targeted by acaricides on A. *mellifera*, and (b) most of the evolutionary history captured by positive selection analyses happened on A. *cerana*, given that coevolution with A. *mellifera* is a relatively recent evolutionary phenomenon, particularly for V. *jacobsoni*.

	V. *destructor* (Vdes_3.0)		**V. *jacobsoni* (Vjacob_1.0)**		**Varroidae**	
	Annotated gene under positive selection	Annotated gene undergoing duplication	Annotated gene under positive selection	Annotated gene undergoing duplication	Annotated gene under positive selection	Annotated gene undergoing duplication
Stress from in-hive temperature						
Heat Shock 70 kDa protein-like [93]		6				
Nutrition						
Guanine metabolism [38]			1			
Metabolic pathways targeted by acaricide						
Glutathione-S-transferase [94]			2			
Sodium channel related [95]	4		2			
Transmembrane proteins	3		4	2		
G-protein coupled receptors	1					
Esterases [96]	2					
GABA-gated ion channels [97]	1				1	
ATP-binding cassette gene family [98]	1		2	2		
Molting and reproduction						
Ecdysteroids pathway with cytochrome P450 [99]			3			
Cuticular protein	2	4		1		
Nutrition and reproduction						
Fatty acid metabolism [100–102]	3					4

525 ## Discussion

In this study, we developed high-quality *de novo* reference genomes and annotations for economically important honey bee parasites V. *destructor* and V. *jacobsoni*. Because V*arroa* mites are, to a large extent, responsible for the global honey bee crisis, and research efforts worldwide target these species, these
530 genomic resources respond to a pressing need [29]. Our analysis revealed that the two V*arroa* species are highly similar genetically, with 99.7% sequence similarity across the nuclear genome. Yet, after the two species have split, they have undergone largely dissimilar evolutionary responses, as evidenced by different patterns of positive selection and gene duplication. Thus, it seems likely that the
535 two species underwent different patterns of adaptation to the same ecological niche, most likely driven by the ancestral host (A. *cerana*).

Inferring what constitutes the ancestral ecological niche of the two mites is key to understanding what selective pressures they faced and how they responded to them. Both V. *destructor* and V. *jacobsoni* are very similar morphologically, and
540 until 20 years ago were mistakenly considered the same species [18]. Their host specificity overlaps as they both parasitize the eastern honey bee A. *cerana* throughout Asia. The only exception reported for the Indonesian A. *nigrocincta* infested by V. *jacobsoni*, though possibly as a result of a recent host switch from A. *cerana* [18,103]. The most common ancestor of V. *destructor* and V. *jacobsoni* might
545 have also been a parasite of the A. *cerana* lineage, and this hypothesis is supported by the host range of V. *underwoodi*, another closely related mite that also parasitizes A. *cerana* in addition to A. *nigrocincta* and A. *nulensis* [104,105]. With no known Varroidae (Mesostigmata) fossils, no information exists about the timing of speciation in V*arroa* mites. Based on an observed C*ox1* gene divergence between V.
550 *destructor* and V. *jacobsoni* of 5.9%, a very rough estimate based on an insect-wide molecular clock (2.3% divergence per Myr) [106] suggests that the two species split ~2-3 Myr. This is after the split between A. *cerana* and A. *mellifera* ranging from 6-9 Myr using C*ox1* and ND2 [107,108] to 17-19 Myr using a combination of mitochondrial,

ribosomal and nuclear *loci* [109]. Thus, the host ranges and molecular estimates suggest that most of the V*arroa* evolution has occurred on the ancestors of the present-day A. *cerana*.

The major ecological difference between these mites is their parapatric distribution (Figure 1). Whether parapatry resulted from their respective biogeographic history, adaptation to different subspecies of A. *cerana*, or is shaped by competition is not known. In fact, even the extent of the geographical overlap between the mite distributions is uncertain, since few surveys actually used molecular markers for identification and most have focused on associations with A. *mellifera* [18,78,105,110]. Yet, parapatry is clearly not due to the inability of the mites to complete their phoretic life stage outside the hive, as host-switched V. *destructor* successfully expanded its range following that of A. *mellifera*. While allopatry could be a result of local adaptation and competitive exclusion, both mites have been reported from the same Thai apiary on A. *cerana* [42,46]. Unfortunately, there are not enough data on A. *cerana* infestation under natural conditions to determine the likelihood that the two V*arroa* species interact and compete. One survey in southern Thailand found 98% parasitism by V. *jacobsoni* under natural conditions, albeit at variable levels of infestation [111], suggesting that encounters between the two species are indeed likely. If the divergent evolutionary between the two V*arroa* species resulted from competition, we predict that long-term coexistence within a hive of A. *cerana* should be unlikely, a hypothesis that we hope will be tested in the future in the zone of sympatry.

In addition, or as an alternative to interspecific competition, it is possible that the divergence between V. *destructor* and V. *jacobsoni* was driven by local adaptation to different A. *cerana* populations. For instance, V. *destructor* was found to infect the "Northern *cerana*", "Himalayan *cerana*" and the "Indo-Chinese *cerana*" morpho-clusters identified by [43] whereas V. *jacobsoni* was reported on the "Indo-Chinese *cerana*" and the "Indo-Malayan *cerana*". These A. *cerana* populations differ in their nesting behaviour, such as the number of combs or even in their body size

[86]. In China alone, A. *cerana* populations were shown to be as genetically divergent for more than 300,000 to 500,000 years (earlier than the recognized level of subspecies for A. *mellifera*) [112]. In addition to genetic differences between the hosts, the climactic environments across Asia are highly variable and could result in local adaptation, though, as mentioned earlier, this did not prevent range expansions after switching hosts (Figure 1). Consequently, the dissimilar selective pressures observed in the mites may have resulted from independent arm-races with different host genotypes under variable environment selective pressures.

Understanding the dynamics of co-infestation by both mites will become important if the host-switched V. *destructor* and V. *jacobsoni* are ever found to co-occur on A. *mellifera*. Presently, host-switched V. *jacobsoni* is restricted to Papua New Guinea, where V. *destructor* is absent [20]. However, if its range expands, it may impose an additional parasitic burden on A. *mellifera*, but whether the effects of this extra parasite are cumulative or whether the parasites will simply locally replace each other is unknown. Our data suggest that if the parasites are able to tolerate each other within the A. *mellifera* colonies, given their different suites of molecular adaptations, they may in fact impose a greater cost than each parasite alone. Clearly, this is another avenue where further experimental work is urgently needed.

Likewise, it is unknown whether chemical treatment options developed for V. *destructor* will also be effective against V. *jacobsoni*. Interestingly, both mite's genomes show signs of selection on a range of genes previously found to be involved in stress and pesticide resistance (Table 2). We hypothesize that these genes were under positive ancestral selection, most likely during the millions of years associated with the A. *cerana* lineage, and could have been co-opted for pesticide resistance more recently. This has been the case with pesticide tolerance in the sheep blowfly (*Lucilia cuprina*), where sequencing of museum specimens has shown that resistance variants pre-dated pesticide use [113]. Similarly, the two-spotted mite (*Tetranychus urticae*) appears to co-opt existing detoxification

machinery in selective response to miticides [114]. However, more detailed population-level work comparing switched and unswitched populations of mites will be necessary to test this hypothesis. Museum specimens or other historical material would be particularly useful in understanding evolutionary trends [115].

Conclusion

Despite the economic importance of brood parasitic mites in A. *mellifera*, it is clear that multiple pressing research avenues still exist concerning their biology, cryptic diversity in the native range, possible geographic ranges of host-switched species, and how co-infestations may proceed. The new reference genomes will allow us to pursue these investigations, develop better tools for V*arroa* control, and to better understand the invasion and evolution of V*arroa* on A. *mellifera*. In particular, we hope that these new resources will greatly benefit future studies to track the population genetic changes and demography of V. *destructor*'s worldwide spread, which has been difficult due to the low polymorphism of previously available markers [110]. More V*arroa spp.* are continuously testing new host [79,81] and may remain undetected. Finally the recent boom in -omics methods for the study of honey bee parasites and diseases underline the need to improve our knowledge on genome structure and organization to investigate the genetic basis behind newly reported acaricide resistance, virulence level or even new host tolerance. Such knowledge combined with the progress in novel manipulation tools, such as RNAi [94,116], as well as more experimental tools like gene drive [117], may provide alternative ways of specifically controlling mite pests without harming honey bees or other insects.

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Data accessibility

V. *destructor* (Vdes_3.0) and V. *jacobsoni* (Vjacob_1.0) assembled reference genomes, and annotation are available on NCBI database respectively with the accession number GCF_002443255 and GCF_002532875. Raw Illumina and PacBio reads are also available in the Sequence Read Archive (SRA) under the Bioprojects PRJDB6279 and PRJNA391052.

Authors' contribution

MAT collected samples for sequencing, carried out the analyses, interpreted the data and wrote the manuscript; RVR carried out the analyses; JMKR collected samples for sequencing; MLG carried out the assembly and quality analyses; SS carried out the computational analyses for Hi-C genome assembly; IL carried out the experimental protocols for Hi-C genome assembly; AKC conceived and designed experiments; JDE conceived and designed experiments; ASM conceived and designed experiments, carried out the assembly and analyses, interpreted the data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

IL and STS are employees and shareholders of Phase Genomics, a company commercializing proximity-ligation technology.

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Figures and Tables

Table 1: Improvement and release of new genome assembly statistics for honey bee *Varroa* mites

Table 2: Genes undergoing positive selection or duplication, which were previously implicated in tolerance to external stress sources and stimuli.

Figure 1: V. *destructor* and V. *jacobsoni* are morphologically similar sister species originally parasitizing the Asian honey bee (A. *cerana*).

Figure 2: Inter and intraspecies mtDNA variability across the genus V*arroa* and host switched lineages.

Figure 3: Positive selection and gene duplication of orthologous genes in parasitic and free-living Acari.

Figure 4: Different genes and pathways under positive selection in the V*arroa* sister species.

Figure 5: Duplicated genes in V. *destructor* are found all along the genome and are involved in different biological pathways.

Supplementary data

Figure S1: Similar genome sizes between the two species of V*arroa* mites estimated from haploid male genome through the k-mer 42 frequency in GenomeScope.

Figure S2: UpSet plot of orthologous genes detected under positive selection detailing the shared number per species.

Figure S3: Treemap plots (REVIGO) analysis for the summary of GO terms in biological processes for gene detected under positive selection when comparing V*arroa* species.

Supplementary table 1: Summary output of ortholog genes and correspondence among the six species included in the phylogeny

Supplementary table 2: GO enrichment of ortholog clusters shared between V*arroa* mites and five other Acari

Supplementary table 3: Genomic details of the 40 genes detected under positive selection in both V*arroa*

Supplementary table 04: Genomic details of the 234 genes detected under positive selection in V. *destructor*

Supplementary table 05: Genomic details of the 225 genes detected under positive selection in V. *jacobsoni* and corresponding location on chromosome of V. *destructor*

Supplementary table 06: GO enrichment analysis of genes detected under positive selection for V. *destructor* and V. *jacobsoni*

Supplementary table 07: Gene duplicated in tandem different between V. *destructor* and V. *jacobsoni*

Supplementary table 08: Gene duplicated in tandem shared by V*arroa* mites and *Tropilaelaps mercedesae*

Supplementary table 09: GO enrichment analysis of genes duplicated only in V. *destructor*

1045 **Supplementary table 10:** GO enrichment analysis of genes duplicated only in V. *jacobsoni*

Supplementary table 11: Gene set enrichment analysis of genes duplicated shared by V*arroa* mites