Divergent selection following speciation in two ectoparasitic honey bee mites

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Abstract

Multispecies host-parasite evolution is common, but how parasites evolve after speciating remains poorly understood. Shared evolutionary history and physiology may propel species along similar evolutionary trajectories whereas pursuing different strategies can reduce competition. We test these scenarios in the economically important association between honey bees and ectoparasitic mites by sequencing the genomes of the sister mite species *Varroa destructor* and *Varroa jacobsoni*. These genomes were closely related, with 99.7% sequence identity. Among the 9,628 orthologous genes, 4.8% showed signs of positive selection in at least one species. Divergent selective trajectories were discovered in conserved chemosensory gene families (IGR, SNMP), and Halloween genes (CYP) involved in moulting and reproduction. However, there was little overlap in these gene sets and associated GO terms, indicating different selective regimes operating on each of the parasites. 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 provide essential resources to survive [3]. Commonly, multiple parasite generations elapse per host generation. Thus parasites are prone to evolve rapidly and may circumvent any gained host advantage [4]. The similar selective regimes acting on phylogenetically unrelated parasites resulted in reproducible evolution such as genome size reduction, functional losses associated with reduced metabolism and adaptive functional gains linked to parasite virulence [5]. Most models of parasite coevolution are limited to interactions between single focal parasites and their hosts, in part because multi-species dynamics are much more complex [6,7]. In nature, the reality is that parasite's success and evolution will also depend on interactions with co-infecting conspecific and heterospecific within its host niche [8–10]. Yet, coevolutionary dynamics involving more than one parasitic species remain poorly understood [11,12].

For example, co-infecting parasites occupying the same host can exclude competitors via direct, or interference competition [13,14]. One strategy to reduce interspecific competition can be spatial separation within regions of the host gut as adopted by cestodes that naturally co-infect sticklebacks [15]. Within the same host, spatial segregation that allows parasite co-existence can ultimately lead to intrahost speciation, as found in gill parasites (*Dactylogyrus*) of freshwater fish (*Cyprinidae*) [16]. Niches may also be partitioned temporally, which allows two fungal sibling species (*Erysiphe quercicola* and *E. alphitoides*) to exploit the same host tree (*Quercus robur*) by reducing direct interference [17]. Alternatively, parasites may shift their strategies to exploit different aspects of the host niche. For example, fire ants (*Solenopsis spp.*) are parasitized by a genus of decapitating phorid fly (*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.) [18]. However, almost nothing is known about 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.

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. Varroa mites are obligatory ectoparasites which feed on fat bodies of developing larvae/pupae and adult honey

bees (shown in *V. destructor*) [19] and reproduce in the brood [20]. Parasitic feeding injures vital the honey bee host but leading to abnormal development [21], reduced worker lifespan [22], the transmission of viruses [23] and pathogenic bacteria [24]. These combined negative effects on individual honey bee decrease the fitness of the colony as a superorganism. Without acaricide treatments, managed western honey bee colonies were reported to decline and die within two to three years [20]. As its name indicates, *V. destructor* is considered as the main devastating force behind colony collapses and impact deeply western honey bee (*A. mellifera*) beekeeping [25,26].

The dramatic effects of *Varroa* mites on *A. mellifera* are due to their recent coevolutionary history. Originally, brood parasitism by *Varroa* ectoparasitic mites has evolved in all *Apis* species except for *Apis mellifera* [27]. Until the 2000s, *V. jacobsoni* and *V. destructor* were considered a single species, based on their life cycle, morphological similarities (Figure 1A) [28] and given that both originally parasitized 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 repeatedly colonized this novel host [29,30] (Figure 1C). In contrast to what has happened to *A. mellifera*, the host-parasite interaction with *A. cerana* is a balanced relationship in which a tolerance "equilibrium" is maintained by a range of parasite adaptations and host counter-adaptations [31–36]. Specifically, the eastern honey bee has impressive host defense traits, including i) self and collective grooming behaviors [37], ii) hygienic behavior by removing mites from infested cell [38], iii) worker brood susceptibility stopping parasite reproduction [33,34], iv) entombing of mites in drone brood and v) shorter worker development cycle limiting mites offspring number [32]. By contrast, most western honey bee populations lack these defensive mechanisms, and hives collapse quickly after *Varroa* infestation. On the part of *Varroa* mites, they evolved highly specialized and adaptive strategies to find, select, feed, reproduce and hide on its host [39]. While many mite adaptations have already been discovered [20,39], the recent use of 'omics approaches has quickly broadened our understanding on *V. destructor* hidden specialization to host [36,40–42]. For example, transcriptomic and proteomic profiling of different *V. destructor* life stages burst the identification of genomic regions related to chemosensory system [40,42,43], reproductive success [41] and linking oogenesis initiation or failure to parasitic reduced metabolism and host defense [44,45].

Here, we ask how both mite species have arrived at this tolerance 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 [46,47]. 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. 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 hybrid *de novo* genome assembly 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.

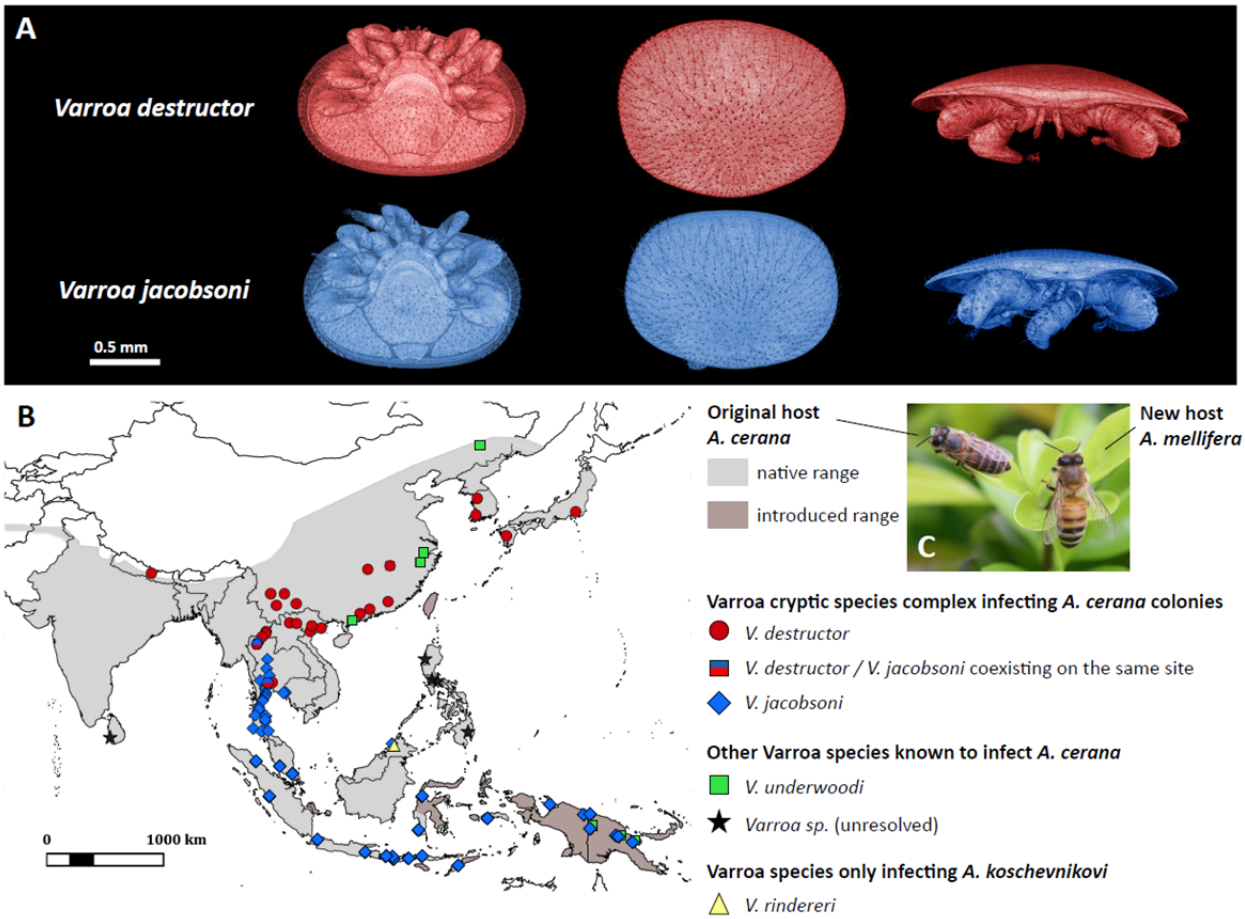


Figure 1: *V. destructor* and *V. jacobsoni* are morphologically similar sister species originally parasitizing the eastern honey bee (*A. cerana*). These species were recognized upon the basis of quantitative morphometric and genetic data [28.] Morphological differences are restricted to differences in body size and shape, as shown in (A) 3D surface models comparison of two fully sclerotized females. (B) Both mites parasitize *A. cerana* as their original host and can coexist in sympatry, occasionally even at the same apiary [46,48], but are mainly parapatric [49]. (C) 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 [30].

141 # Methods

142

143 **Sequencing strategy and *Varroa* mite collection**

144

145 We took advantage of the haplodiploid sex determination of both *V. destructor* and *V.*
146 *jacobsoni* to generate data to reduce the ambiguity due to heterozygous sites for a high
147 contiguous hybrid genome assembly. The sequencing strategy adopted in this study combined
148 shorts reads (Illumina) for which input DNA can be obtained from a single mite individual, long
149 reads of tens of kilobases (PacBio) which requires a large quantity of high-molecular-weight
150 DNA and Hi-C to capture chromosome conformation and interactions.

151 Haploid males (~0.7 mm by 0.7 mm) are only found inside capped brood cells after
152 initiation of reproduction while diploid females (~1 mm by 1.5 mm) can be found in the brood
153 and on adult honey bees. All *V. destructor* samples were collected from *A. mellifera* colonies of
154 Okinawa Institute of Science and Technology (OIST) experimental apiary in Japan (26.477 N
155 127.835 E) in August-September 2016. One mature male *V. destructor* was collected within a
156 worker cell at the red-eye pupa developmental stage (day 15 to 16) using a brush and kept in
157 absolute ethanol at -80◦ C. Mature adult females were collected from adult honey bee
158 workers. In order to obtain a large number of mites infecting adult workers, we modified the
159 standard powdered sugar method [50] for an entire managed colony as follows: one hive box
160 containing three to four frames with honey bee workers and no queen was placed on top of a
161 collecting white tray; approximately 500g of icing sugar was then applied using sieve filter
162 powder strainer; the sugar and honey bees were shaken in the tray for 2 min and then
163 separated using a sieve filter, placing the sugared-workers directly within the hive. This process
164 was repeated on adjacent colonies. *Varroa* mites were separated from their hosts and trapped
165 in the icing sugar. Following a water rinse on a gauze mesh, a total of 1,207 alive *Varroa*
166 females were snap frozen at -80◦ C until laboratory processing (inactive, sluggish or dead mites
167 were discarded). Samples of *V. jacobsoni* were obtained from its first detection survey where it
168 was found reproducing in the western honey bee [30]. One mature male (VJ856) collected from *A.*
169 *mellifera* drone brood cell in Papua New Guinea (between EHP and Henganofi border,
170 30/05/2008) was used to prepare a whole genome library.

171 One sclerotized mature female *V. destructor* (VDOKI-01) collected from OIST apiary in
172 the reproductive phase and one sclerotized mature female *V. jacobsoni* (VJ347-11) collected
173 from *A. cerana* originating in Java (Cililin central Java, 13/06/1998 by D. Anderson) were
174 processed for X-ray microtomography (micro-CT) using a Zeiss Xradia 510 Versa scanner. 3D
175 surface models were reconstructed from micro-CT outputs using Amira software to visualize

176 the slight morphological differences when focusing on idiosoma ventral and dorsal views and
177 whole body in profile.
178
179 **DNA extraction, genome sequencing, assembly, and annotation**
180
181 Genomic DNA was extracted from each single haploid male *V. jacobsoni* and *V.*
182 *destructor* using QIAamp DNA Micro Kit (Qiagen) following manufacturer' s instructions. The
183 total amount of dsDNA was measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit
184 (Invitrogen). Short-insert of 250 bp paired-end libraries were prepared for both individuals
185 using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (Biolabs) and 10 PCR cycles. Size-
186 selection and cleanup was applied using SPRI beads and library was subsequently checked using
187 a Bioanalyzer High Sensitivity DNA kit (Agilent). Both paired-end libraries were each sequenced
188 on two lanes of Illumina HiSeq 2500 at the OIST sequencing center in paired-end 250 cycle
189 mode.
190 Prior to assembly paired-end reads were de-duplicated using ParDRe [51], and host and
191 microbial contamination was filtered using DeConSeq (parameters: -i 97 -c 95) [52], screening
192 against genomes of *Apis cerana*, *Apis mellifera*, as well as bacterial genomes and genomes of
193 bee diseases and parasites. The cleaned reads were then merged using PEAR (parameters: --
194 min-overlap 10 --memory 80G --threads 10 -n 200 -m 600 -p 0.0001) [53] and assembled using
195 Newbler (v. 2.6) (parameters: -large -m -cpu 24 -mi 98 -siom 390 -l 1000 -a 500 -urt -novs -a
196 1000 -minlen 45 -ml 100) [54]. The contigs were then scaffolded using publicly available RNA-seq
197 data assembled using Trinity [55,56]. For *V. destructor* further scaffolding was carried out using Hi-
198 C, performed by Phase Genomics. Hi-C read pairs were aligned to the initial assembly using
199 BWA-MEM with the "-5" option [57], keeping only primary properly paired alignments (samtools
200 with the "-F 2316" filter [58]). Scaffolding was then performed using Phase Genomics' Proximo Hi-
201 C scaffolding pipeline [59], which builds on the LACHESIS method [60].
202 To estimate genome characteristics, K-mers from cleaned reads were counted using
203 jellyfish version 2.2.7 [61] for the following values of K: 19, 25, 31 and 42 and coverage cut-off of
204 1,000. Frequencies were only computed on HiSeq 2500 reads from one individual haploid male
205 of each species. Computed and selected K-mer histograms for k=42 were analyzed with
206 GenomeScope to estimate genome size, kcoverage, number of duplicates [62]. We used the
207 bioinformatically derived genome sizes for genome coverage estimation.
208 Small batches of adult females *V. destructor* (1,207 mites total) were pooled and
209 crushed by pestle in 1.5 mL Eppendorf tubes. The powder was then lysed using proteinase K
210 and lysis buffer at 56□ for 20 min. Sample lysate was thus transferred to a Maxwell Cartridge.

211 The remaining step was fully performed on the Maxwell 16 automated purification system
212 (Promega) with the LEV Blood DNA mode. Library preparation for PacBio RSII sequencing was
213 performed using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) following the
214 manufacturer's recommendations. SMRTbell libraries were prepared and sequenced at the
215 OIST sequencing center on 48 SMRT cells. These reads were trimmed using proovread [63] and
216 then used to gap-fill the Hi-C scaffolded *V. destructor* assembly using PBJelly (parameters: -
217 minMatch 8 -minPctIdentity 70 -bestn 1 -nCandidates 20 -maxScore -500 -nproc 24 -
218 noSplitSubreads) (v. 15.8.24) [64].
219 Annotation for both genomes Vdes_3.0 and Vjacob_1.0 was carried out by NCBI using
220 NCBI's automated genome annotation pipeline. This pipeline takes advantage of species-
221 specific RNA-seq data, as well as extensive protein homology data stored in GenBank [65].
222
223 **Identification of *Varroa* mite strains using mtDNA and sequence similarity**
224
225 Most previous taxonomic work relied on mitochondrial fragments. We used
226 mitochondrial analysis to determine and compare the lineages of *Varroa* species used for
227 genome sequencing to the previously described haplotypes. The whole mitogenome of *V.*
228 *destructor* (accession NC_004454.2 [66,67]) was available from the NCBI database and used as a
229 reference for read mapping. Raw Illumina® reads for both *V. destructor* and *V. jacobsoni* males
230 were mapped onto the entire reference sequence (16,476 bp) using NextGenMap 0.5.0 [68].
231 Mapped reads were sorted and PCR duplicates were removed using Samtools 1.3.1 [58].
232 Maximum coverage of 500x was subsampled on all mapped reads using VariantBam 1.4.3 [69].
233 Subsequently, variant calling was performed using FreeBayes v1.1.0-46 [70], with the following
234 parameters others than the default: minimum mapping quality of 20 and minimum base quality
235 of 5. Similarly, Illumina reads used to construct Vjacob_1.0 were mapped against the reference
236 Vdes_3.0 to detect the number of variants in the nuclear genome. Consensus mitochondrial
237 sequence was generated for each individual using vcf2fasta from Vcflib library [71].
238 One standard method to identify Varroa species and haplogroup is to sequence a partial
239 region of the *Cox1* gene (426 bp) from the mitochondrial DNA [50]. The same region was
240 extracted from both Vdes_3.0 and Vjacob_1.0 generated mitochondrial sequence and aligned
241 together with 26 *Varroa spp.* haplotypes previously described [28,72–74]. Given its worldwide bee
242 pest status, *V. destructor* haplogroups were better described than *V. jacobsoni* and additional
243 larger *Cox1, Cox3, Atp6* and *cytB* mitochondrial sequences were available on NCBI. Therefore, a
244 more resolutive comparison within *V. destructor* was done by aligning concatenated sequences
245 of the four genes with 22 haplotypes described previously [72,75]. Sequences were aligned using

246 ClustalW, checked by hand and a consensus maximum likelihood tree was constructed after
247 5000 bootstraps in Mega 7.0.18 [76].
248 A distribution map of the *Varroa* mites infecting *A. cerana* was built from reviewing
249 literature. Conservatively, only the presence of *Varroa spp.* confirmed by mtDNA barcoding
250 after the taxonomic revision was plotted [28,46,72–74,77–79] onto the potential native and introduced
251 range of the Asian honey bee host [47,80]. The map was produced using QGIS 2.16.1 (Open Source
252 Geospatial Foundation Project qgis.osgeo.org) and the public domain natural 10m earth raster
253 NE1_HR_LC github.com/nvkelso/natural-earth-raster). An additional interactive map showing
254 the reported distribution of the 26 mtDNA haplotypes is available at
255 maevatecher.github.io/varroa-denovo-genomes with layers control options.
256
257 **Prediction of gene orthology, selection, and duplication in *Varroa* mites**
258
259 The Orthonome pipeline (www.orthonome.com) [81] was applied to predict orthologues
260 (pairwise and 1:1 orthogroups), inparalogues and lineage-specific gene expansions from the
261 two parasitic *Varroa* mites' genomes sequenced and annotated here and four Acari annotated
262 reference genomes available from NCBI
263 (www.ncbi.nlm.nih.gov/genome/browse/#!/overview/Acari). The Orthonome pipeline uses
264 phylogenetic information and microsynteny to identify gene copy number changes and tandem
265 duplications between pairs of species using the MSOAR2.0 pipeline [82]. We included four
266 outgroups which had fully sequenced genomes, including the eastern honey bee ectoparasitic
267 mite *Tropilaelaps mercedesae* [83], the parasitic tick *Ixodes scapularis* [84]; the free-living Western
268 predatory mite *Metaseiulus occidentalis* [85] and the free-living two spotted mites *Tetranychus*
269 *urticae* [86]. Total and pairwise/lineage intersection of orthologs gene sets were represented
270 using Upset plots [87] using R. The 1:1 orthogroups with both *Varroa* species represented and > 3
271 species were used for positive selection hypothesis tested on phylogeny using HyPhy to
272 calculate the mean dN/dS and test using aBSREL (adaptive Branch-Site Random Effects
273 Likelihood) [88]. This test allowed estimation of selection along specific branches of the tree,
274 including ancestral branches. Only branches with a significant p-value (< 0.05) after FDR
275 corrections were considered as positively selected in each orthogroup. Additionally, tandem
276 duplications in the three parasitic mites were identified from inparalogues in immediate vicinity
277 of the orthologues. All orthologous genes in Varroa mites under positive selection and/or
278 duplicated in tandem were checked by hand by using BLAST. Duplication events with more than
279 two genes were systematically checked by aligning the annotated coding sequence (CDS) with

280 ClustalW alignment implemented in Geneious R8 (Biomatters, Ltd., Auckland, New Zealand) and
281 construct a tree with IQ-TREE [89].
282
283 Gene ontology (GO) enrichment analysis of the positively selected genes in honey bee
284 parasitic mites were carried out using the GOstats R package [90]. The same process was applied
285 for gene duplication in *Varroa* mites. Biological processes associated with these GO terms were
286 summarized and visualized using REVIGO [91] (http://revigo.irb.hr). The semantic similarity was
287 measured using the Resnik's measure (SimRel) [92] and the threshold used was C = 0.5 (i.e. small).
288 The results were then used to produce a scatter plot and treemap plot using the ggplot2
289 package. The same package was used to plot the localization of the genes under positive
290 selection and/or duplicated onto *V. destructor* chromosomal scaffolds. Significance of overlaps
291 between gene sets and associated GO terms were tested using Fisher's exact test with
292 GeneOverlap R package. Code and raw plots for exploratory analysis of positively selected
293 genes, duplication in tandem and GO term association are available online
294 (maevatecher.github.io/varroa-denovo-genomes).
295
296
297 **Chemosensory and ecdysteroid pathway-related gene families in host-parasite interactions**
298
299 Varroa mites are blind and rely on their chemosensory system to find a suitable host
300 within a densely packed honey bee colony [39]. Chemical cues are transported to the nervous
301 system by binding to soluble carrier proteins and membrane proteins in insects. Several
302 homologous chemosensory protein families involved in these pathways have been identified in
303 Acari and *Varroa* mites [40,42,43,93]. Here we used the available catalogue of protein sequences
304 reported from *V. destructor* forelegs transcripts [43,93] containing six Odorant Binding Proteins
305 (OBP), six Niemann-Pick disease protein, type C2 (NPC2), seven Sensory Neuron Membrane
306 Proteins (SNMPs), three Glutamate Receptors (GRs) and 17 Ionotropic Glutamate Receptor
307 (IGRs). We identified these proteins in both *V. destructor* and *V. jacobsoni* annotated genomes
308 using BLASTP and compiled predicted protein sequences (keeping one isoform if several exist
309 for the same gene). We then aligned each family of protein sequences with other sequences
310 from other Acari and insects using MAFFT version 7 [94] (parameters: E-INS-i, the BLOSUM62
311 matrix, and MAFFT-homolog option with 100 homologs, E threshold = 0.1). We used IQ-TREE [95]
312 to find the best substitution model for each family alignment, with state frequency estimated
313 by maximum likelihood, 1000 ultra-fast bootstraps, 1000 replicate for SH-aLRT branch test. All
314 generated phylogenetic trees were visualized and exported using iTOL v4 [96].
315

316 We ran the same process for the cytochrome P450 enzyme (CYP) genes (including the
317 Halloween genes), known to be involved in the steroid molting hormone ecdysone biosynthesis
318 pathway in Arthropods. Ecdysteroids derivatives are also important as they are involved in the
319 vitellogenin production and oogenesis initiation in *V. destructor* after feeding on its host[44,97]. To
320 assess which CYP genes and in particular the Halloween genes were present in both *V.*
321 *destructor* and *V. jacobsoni* genomes*,* we used sequences from the fruit fly *Drosophila*
322 *melanogaster*, the mosquito *Aedes aegypti*, both honey bee hosts *A. mellifera* and *A. cerana*, as
323 well as other Acari (same name code as in a previous study on *M. occidentalis* [98]).
324
325

Results

Two new reference genomes for *V. destructor* and *V. jacobsoni*

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. PacBio sequencing yielded 20.3 Gb of *V. destructor* reads with an N50 of 13.5 kb (66× coverage), which was used to fill 84.4% of the gaps in the scaffolded HiC assembly. Final *de novo* assemblies were named Vdes_3.0 (GCA_002443255.1) and Vjacob_1.0 (GCA_002532875.1) and are available on NCBI.

Through the k-mer analysis, the genome size estimates ranged from 369.02 to 365.13 Mbp using k = 42 (Figure S1). Both Vdes_3.0 and Vjacob_1.0 final assembly size were close to the predicted estimates but *V. jacobsoni* genome size was slightly shorter than *V. destructor* with 365.59 Mbp < 368.94 Mbp, respectively (Table 1). Previous assemblies size and contiguity for *V. destructor* with Vdes_1.0 [99] and Vdes_2.0 were improved as detailed in Table 1. 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. When comparing to invertebrate assemblies, Vdes_3.0 genome is among the most highly contiguous with to date the best-reported scaffold N50 and best contig N50 for Acari and Mesostigmata as reported on the i5k database (i5k.github.io/arthropod_genomes_at_ncbi) [100].

Using Hi-C data, seven major scaffolds were constructed for *V. destructor* 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 [101]. Contrary to Vdes_3.0 which benefited from long reads and chromosome capture sequencing, *V. jacobsoni* Vjacob_1.0 genome was assembled from 8,241 contigs into 4,881 scaffolds with an N50 of 233,810 bp. Similar GC content composed 40.9% were found for both sister species Vdes_3.0 and Vjacob_1.0. The automated NCBI Eukaryote annotation pipeline predicted 10,247 protein-coding genes (out of 12,849 genes and pseudo-genes) for Vdes_3.0, and 10,739 protein-coding genes (out of 15,486 genes and pseudo-genes) for Vjacob_1.0 [65] (based upon transcriptome data available on the database in October 2017).

When Vjacob_1.0 reads were mapped onto the Vdes_3.0 reference genome, a total of 1,125,230 biallelic, filtered SNPs were detected. Relative to Vdes_3.0 genome size, sequence divergence with Vjacob_1.0 amounted to 0.30% across the nuclear genome sequence.

360 **Table 1: Statistics for the release of new genome assembly for honey bee *Varroa* mites and**
361 **improvement from the first *V. destructor* draft genome.**
362

		V. destructor			*V. jacobsoni*
Genome assembly		**BRL_Vdes_1.0**	**Vdes2.0**	**Vdes_3.0**	**Vjacob_1.0**
NCBI accession		GCA_000181155.1	GCA_000181155.2	GCA_002443255.1	GCA_002532875.1
	Assembly size (Mb)	294.13	331.92	368.94	365.59
	Coverage	5x	60x	119x	57x
	Gap size (bp)	0	2,821,918	271,335	408,908
	GC content (%)	-	40.1	40.9	40.9
Scaffold	Scaffolds (n)	-	20,448	1,426	4,881
	N50 scaffold length (bp)	-	128,078	58,536,683	233,810
	L50 scaffold length (bp)	-	703	3	482
Contig	Contigs (n)	184,190	52,152	4,498	8,241
	N50 contig length (bp)	2,262	15,568	201,886	96,009
	L50 contig length (bp)	40,912	6,465	556	1,168
Annotation	Gene number (n)	-	-	12,849	15,486
	Mean length (bp)	-	-	18,634	14,657
	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
	Intron number (n)	-	-	94,154	93,749
	Mean intron length (bp)	-	-	3,299	2,898
	Exons number (n)	-	-	110,06	111,624
	Mean exons length (bp)	-	-	537	487
Source		from Cornman *et al.*(2010) BMC Genomics		This study	This study

363
364

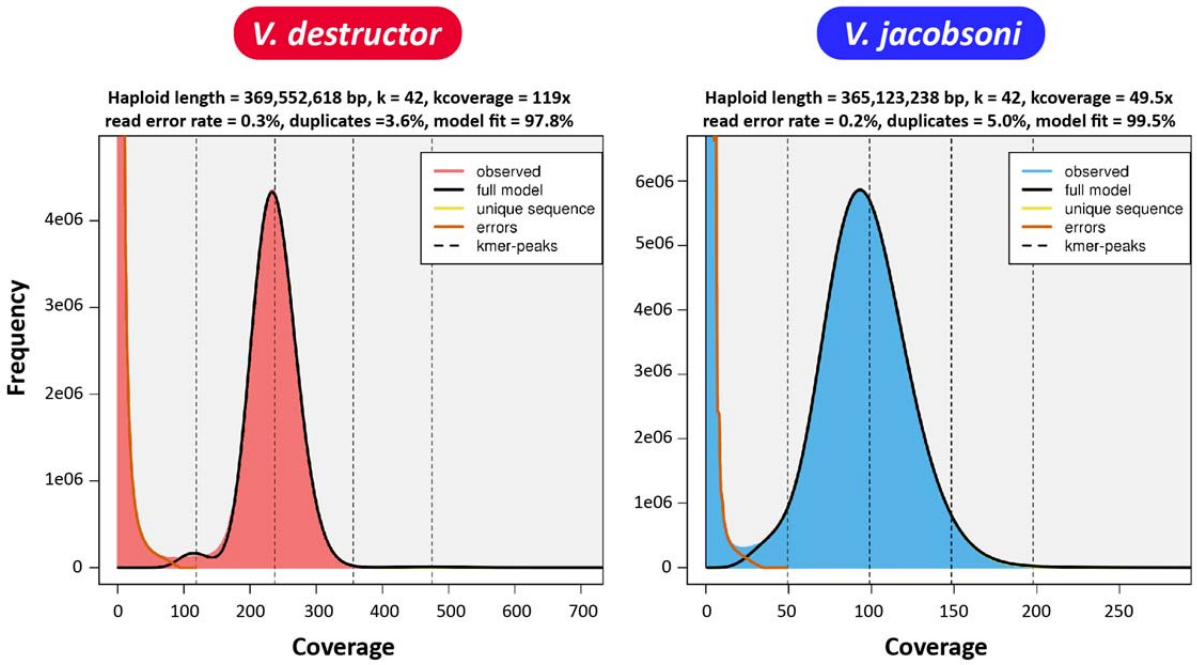


Figure S1: Similar genome sizes between the two species of *Varroa* mites estimated from haploid male genome through the k-mer 42 frequency in GenomeScope.

Identification and confirmation of *Varroa* mite species and mtDNA lineages

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) [66,67] (Figure 2A). Despite presenting similar morphological phenotypes mainly distinguishable through female body size [28,50] (Figure 1A), the *V. jacobsoni* mitochondrial genome diverged from Vdes_3.0 mtDNA at 5.1% of all nucleotides.

Given that *Cox1* is a standard marker used to identify the *Varroa* mite "haplogroup", this region was extracted and realigned with 26 described sequences from native and introduced populations. Divergence within the complete *Cox1* sequences of the *V. destructor* and *V. jacobsoni* reference genomes rose up to 5.9%. The partial *Cox1* sequence (426 bp) of Vdes_3.0 clustered with *V. destructor* sequences and was clearly 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 the Vdes_3.0 mitochondrial genome was identical to that of the invasive Korean K1-1/K1-2 sequence (Figure 2C).

By contrast, the *V. jacobsoni* (Vjacob_1.0 i) partial *Cox1* region (426 bp) was a unique new haplotype with 99% nucleotide similarity to Java (one transition point mutation G/A) and Bali (two transitions) haplotypes and ii) the concatenate sequence (only *Cox1, Atp6,* and *Cox3*)

was also a distinct haplotype from the only referenced *V. jacobsoni* Laos 1 and 2. We named Vjacob_1.0 mtDNA haplotype as Papua New Guinea, which has been formerly detected in field surveys and reported in host switch events on *A. mellifera* (Roberts, unpublished data).

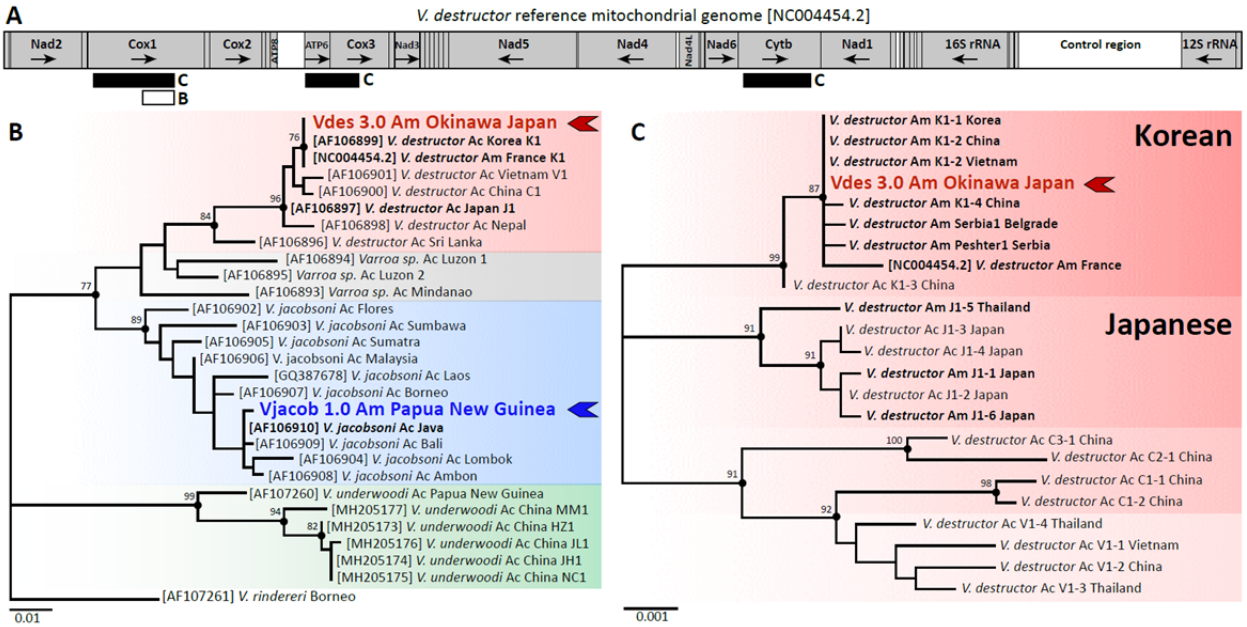


Figure 2: Inter and intraspecies mtDNA variability across the genus *Varroa* and host switched lineages. Different mitochondrial markers used in the literature to discriminate the four *Varroa* species (and unresolved one) based on partial *Cox1* [28] (A,B) and intraspecies lineages using larger *Cox1, ATP6-Cox3* and *CytB* regions [72,75] (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* mite partial *Cox1* sequences (B). Variability of *V. jacobsoni* is higher than that of 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 [30] (B). Several haplotypes from the Korean and Japanese lineages successfully jumped on *A. mellifera* (bold) [72,75] but only K1-1/K1-2 is quasi-cosmopolitan and can even be retrieved in the native range of the Japanese lineage as illustrated by its presence in Okinawa (red arrow) (C). Ac: identified from *Apis cerana*, Am: identified from *Apis mellifera.*

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

The arthropod genome database at NCBI presently contains only two other published representative Mesostigmata genomes: the brood parasitic honey bee mite *Tropilaelaps mercedesae* (GCA_002081605.1) [83] and the non-parasitic Western predatory mite *Metaseiulus*

411 *(= Typhlodromus or Galendromus) occidentalis* (GCA_000255335.1)[102]. Orthologue prediction
412 was performed by comparing annotated *Varroa* genes to those of the two other Mesostigmata
413 species gene sets and those of two additional Acari: the parasitic tick *Ixodes scapularis*
414 (GCA_000208615.1) and the free-living two-spotted mite *Tetranychus urticae*
415 (GCA_000239435.1). The tick presents the largest set of genes (n = 20,486) and also possessing
416 a haploid genome more than four times larger 1.76 Gb [84]. Orthology prediction analysis using
417 the six genomes clustered the genes by domain into 7,502 super-orthologue groups consisting
418 of orthologues and in-paralogues [81]. A total of 11,123 orthologue groups were predicted and
419 42.8% were shared by the five parasitiformes and the outgroup spider mite *Tetranychus*
420 (Supplementary Table 1). The concatenated phylogenetic tree built on the genomic sets based
421 on 4,758 genes using IQ-TREE [89] confirmed the close relationship between the three mite
422 species compared to other Acari (Figure 3). The honey bee ectoparasitic mites, *Varroa* and
423 *Tropilaelaps* shared 813 lineage-specific orthogroups (akin to gene families) of which 65.9%
424 were Varroidae-lineage-specific. The two sibling *Varroa* species shared 9,628 orthologous genes
425 including 536 which were private to Varroidae.
426 Orthologous genes specific to the Parasitiformes ancestral branch (*i.e.*, before *T. urticae*
427 and other Acari species split) was enriched for molecular functions and biological processes
428 involved in several transporters and channel activities (Supplementary Table 2). When
429 considering the Mesostigmata ancestral branch (*i.e.*, before *M. occidentalis* and the three
430 honey bee parasites species split), GO terms related with structural constituents of the cuticle
431 were the most represented. At the Varroidae ancestral branch level (*i.e.*, before the two species
432 split), orthologous genes showed GO terms mainly related to endonuclease activity.
433
434

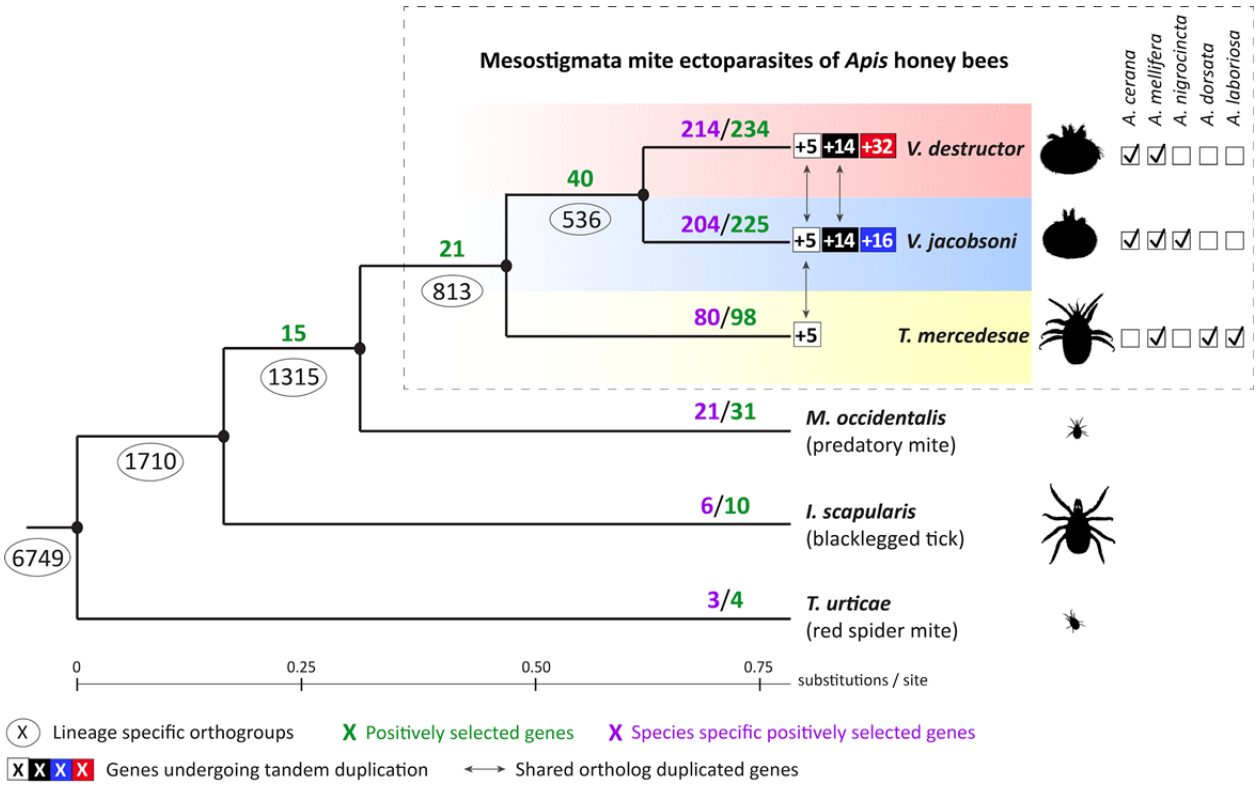


Figure 3: Positive selection and gene duplication of orthologous genes in parasitic and free-living Acari. For the honey bee mite parasites, host ranges are shown as checkboxes and individual records had to meet the following conditions: 1) Mites had to have been found reproducing or observed in bee colonies in independent surveys, and 2) the identity of cryptic *Tropilaelaps* and *Varroa* species was confirmed by molecular markers (e.g. *Cox1* barcoding [28,103]). The number of orthologous genes in each lineage is circled at the nodes. The number of positively selected genes for each branch is shown in green, with the number of species-specific genes shown in purple. Gene duplications within honey bee parasitic mites are shown in boxes: white squares = shared genes, black squares = *Varroa*-specific genes by, red and blue squares = *V. destructor* and V. *jacobsoni* specific genes, respectively. 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 [28,50]. Despite sharing the same ancestral host, the *Varroa* sister species show different patterns of adaptive molecular evolution and exhibit different gene expansion patterns, suggesting different evolutionary trajectories.

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

A total of 234 and 225 genes were detected under positive selection for *V. destructor* and *V. jacobsoni* branches, respectively, though only 13 were shared among this list (2.8%)

456 (Fisher's exact test p = 0.003) (Figure 3). Additionally, 40 genes were detected under positive
457 selection when looking at the Varroa ancestral branch, *i.e.*, before the two species split. On
458 these set combined, the sibling species *V. destructor* and *V. jacobsoni* presented 81.0% and
459 80.4% species-specific genes with positive selection signals, respectively (Figure S2,
460 Supplementary Table S3). The remaining proportion of positively selected genes shared
461 involved the other honey bee mite *T. mercedesae* rather than with any other Acari. The
462 genomic regions associated with genes under positive selection were found and distributed
463 along all major chromosomes of *V. destructor*, and, assuming preservation of synteny, in *V.*
464 *jacobsoni* as well (Figure 4A).

465 Semantic distribution of the GO terms for the genes under positive selection in each
466 *Varroa* species showed non-significant overlap (p = 0.385 > 0.050). The few terms that
467 overlapped involved biological processes in essential functions such as the molting cycle -
468 chitin-based, fatty acid, and lipid metabolism and responses to pH (Figure 4B). More
469 specifically, chitin is the main constituent of the mite cuticle hardening it against both bees and
470 the environment. Its metabolism varies throughout the mite life cycle, especially during
471 reproduction or molting [41]. Fatty acid metabolism could be related to the feeding on the fat
472 body on the host, as well as oogenesis. Although few overlaps exists when GO terms list were
473 reduced using Resnik's measure, the REVIGO scatterplot and treemap visually suggest
474 functionally different routes to host adaptation (Figure 4B, Supplementary Table S4). In *V.*
475 *destructor*, GO terms were mainly associated with 1) regulation of membrane depolarization, 2)
476 retinal cone cell development and myofibroblast differentiation, 3) mRNA cis splicing via
477 spliceosome, 4) cellular response to alcohol (i.e., any process that results in a change or activity
478 of a cell) whereas GO terms in *V. jacobsoni* related to 1) protein processing involved in targeting
479 to mitochondria, 2) vitamin K metabolic process, 3) response to pH, 4) gonad development l
480 and other processes detailed in (Figure S3AB).

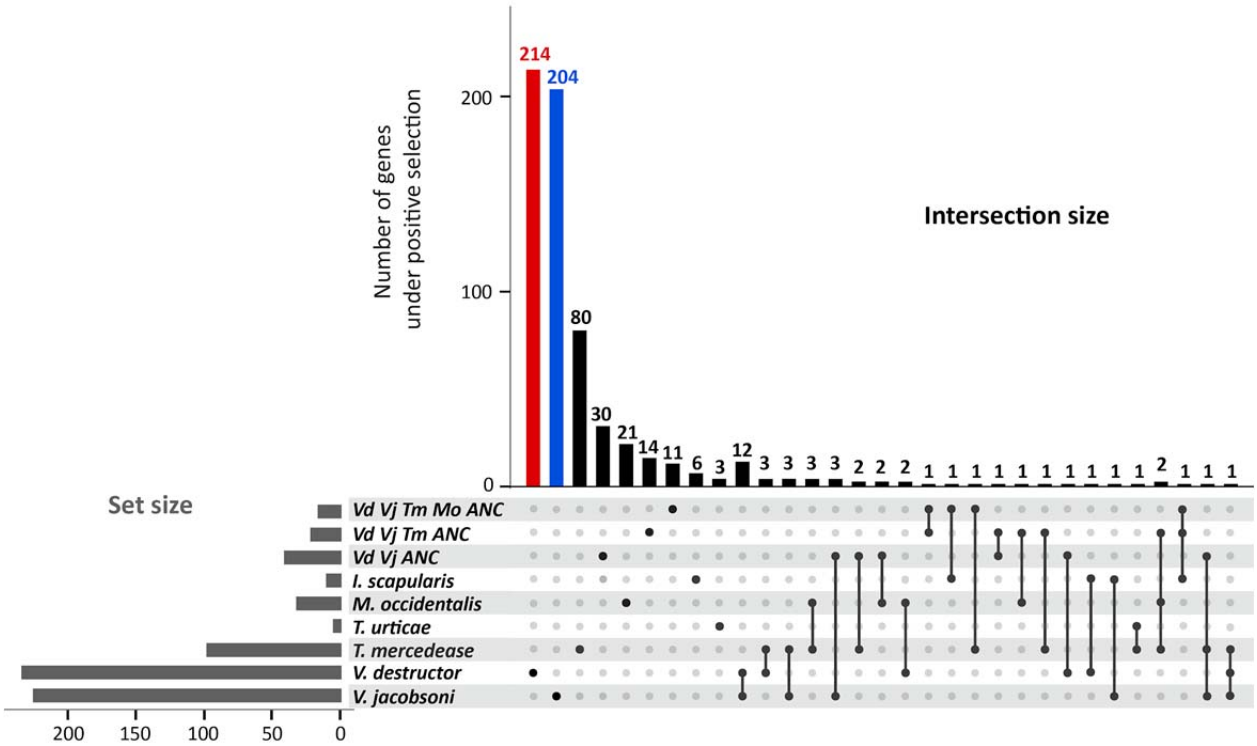
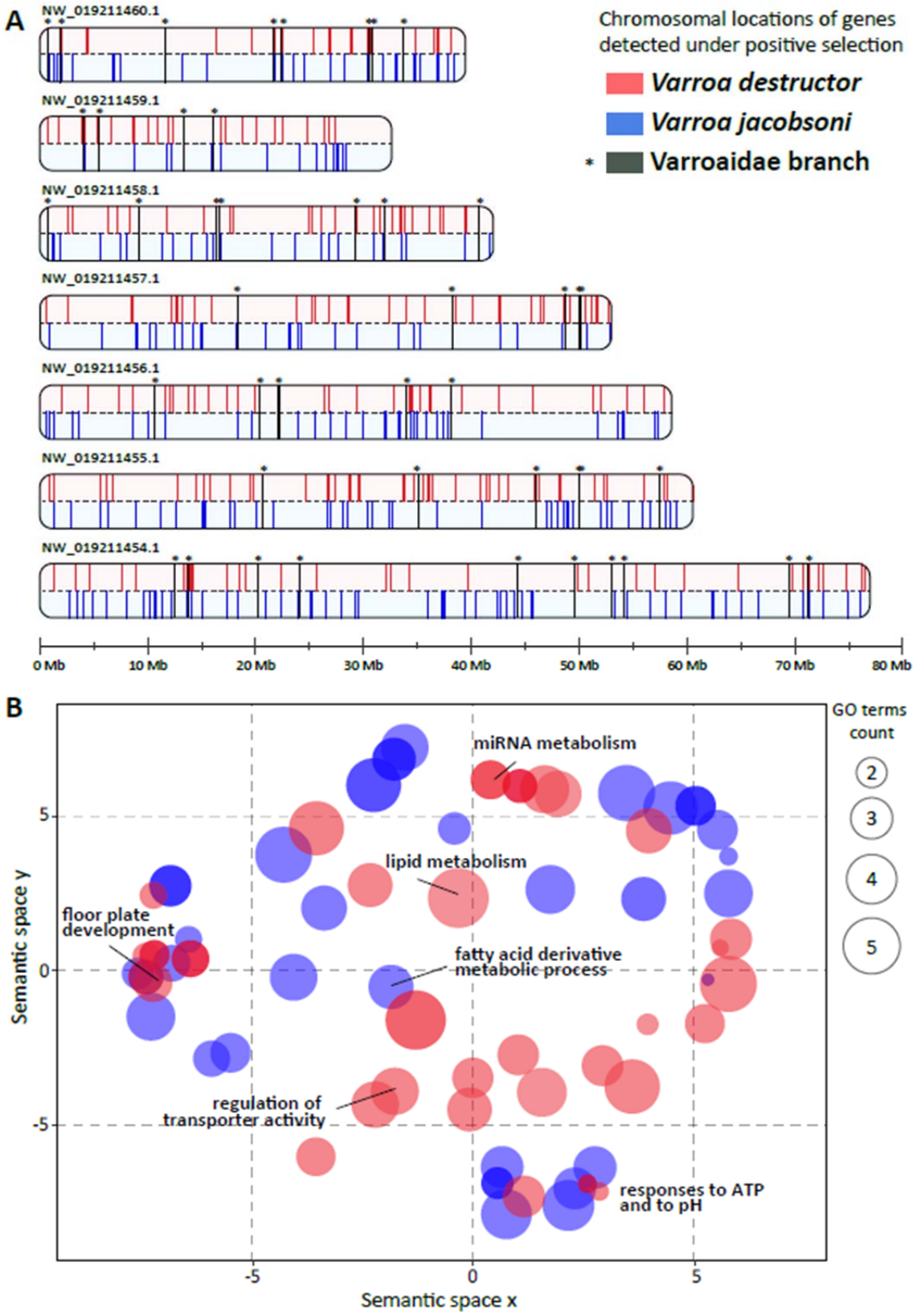


Figure S2: UpSet plot of orthologous genes detected under positive selection detailing the shared number per species. Vd Vj Tm Mo ANC = genes shared with the ancestral and modern Mesostigmata (*V. destructor, V. jacobsoni, T. mercedesae,* and *M. occidentalis*), Vd Vj Tm ANC = set of genes shared with the ancestor of Asian mites and Vd Vj ANC = set of genes shared with the ancestral Varroidae.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NW_019211460.1
NW_019211459.1
NW_019211458.1
NW_019211457.1
NW_019211456.1
NW_019211455.1
NW_019211454.1
Chromosomal locations of genes detected under positive selection
Varroa destructor
Varroa jacobsoni
* Varroaidae branch
0 Mb
10 Mb
20 Mb
30 Mb
40 Mb
50 Mb
60 Mb
70 Mb
80 Mb
B
GO terms count
2
3
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5
miRNA metabolism
lipid metabolism
floor plate development
fatty acid derivative metabolic process
regulation of transporter activity
responses to ATP and to pH
Semantic space y
Semantic space x
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0
-5
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488
489

490 **Figure 4: Genes and pathways under positive selection in the *Varroa* sister species.** (A) Red and blue
491 bars (in 5 kb windows) represent locations of genes in *V. destructor* and *V. jacobsoni*, respectively. *V.*
492 *jacobsoni* data are mapped to *V. destructor* scaffold positions for comparison. Black asterisks indicated
493 genes under positive selection, shared by both species (n = 12) and detected in the *Varroa* ancestral
494 lineage prior to the split of the two species (n = 40). (B) Semantic space analysis of significantly enriched
495 GO terms (BP, CC, and MF) over-represented in genes detected under positive selection for *Varroa* mites
496 Bubble color indicates the species for which GO terms were enriched (all p-value < 0.05) and size
497 indicates the frequency of the GO term found in the GOA database. There was little overlap in analyses
498 at both gene (2.8%) and functional levels (0.8%), suggesting different selective pressures on the two
499 sister species since they split.
500

Figure S3: Treemap plots (REVIGO) analysis for the summary of GO terms in biological processes for gene detected under positive selection when comparing *Varroa* species. Related GO terms were joined in clusters (color) and the size of the rectangles was adjusted to reflect the p-value and with a semantic similarity (C = 0.5).

508 Tandem duplication was detected in 51 genes for *V. destructor*, 35 in *V. jacobsoni* and
509 70 in *T. mercedesae* (Figure 3). Numbers of duplication events and gene clusters within a
510 species varied from two to six units and generally involved adjacent chromosomal locations in
511 *V. destructor* (Figure 5A, Supplementary Table S5). In duplicated genes found only in *V.*
512 *jacobsoni*, 23 had no orthologs in the *V. destructor* genome. However, most duplications are on
513 the same scaffold with the exception of the ABC gene family where genes are present on two
514 scaffolds (online supplemental data). Manual checks of duplicated clusters with more than two
515 genes validated all of the duplications except for the Histone 2A family (vd.dup.gene_01) where
516 gene LOC111255102 located on the minor scaffold BEIS01000444.1 was removed since it is
517 100% identical to LOC111250948 located on chromosome 1 (BEIS01000001.1) and may be an
518 artefact of mis-assembly.
519 Only five duplicated gene clusters were shared by all three honey bee parasite species.
520 These amounted to 9.8% and 14.3% of detected tandem gene duplications for *V. destructor* and
521 *V. jacobsoni*, respectively (Figure 2, Supplementary Table S6). GO terms associated with these
522 duplicated gene clusters related to chitin, amino sugars, and drug and carbohydrate derivatives.
523 Conversely, the two *Varroa* sister species shared 14 gene clusters undergoing tandem
524 duplication, representing 27.5% and 40.0% of the total size for *V. destructor* and *V. jacobsoni*,
525 respectively. When summarizing the list of duplicated shared genes by both *Varroa* species, GO
526 terms were associated with biological processes in glycerophospholipid catabolism, body
527 development, regulation of DNA-templated transcription/elongation and amino-sugar
528 metabolism (Supplementary Table S7). Finally, 62.7% of the duplicated genes were exclusive to
529 *V. destructor* whereas 45.7% were only detected in *V. jacobsoni*. Genes duplicated in *V.*
530 *destructor* were associated with GO terms related to biological processes such as
531 mechanical/external stimulus, regulation of endoribonuclease activity and phagocytosis (Figure
532 5B, Supplementary Table S7-8). In contrast, genes duplicated in *V. jacobsoni* were involved in
533 biological processes of the skeletal myofibril assembly, Golgi calcium ion transport, and striated
534 muscle contraction (Figure 5B, Supplementary Table S7-8).
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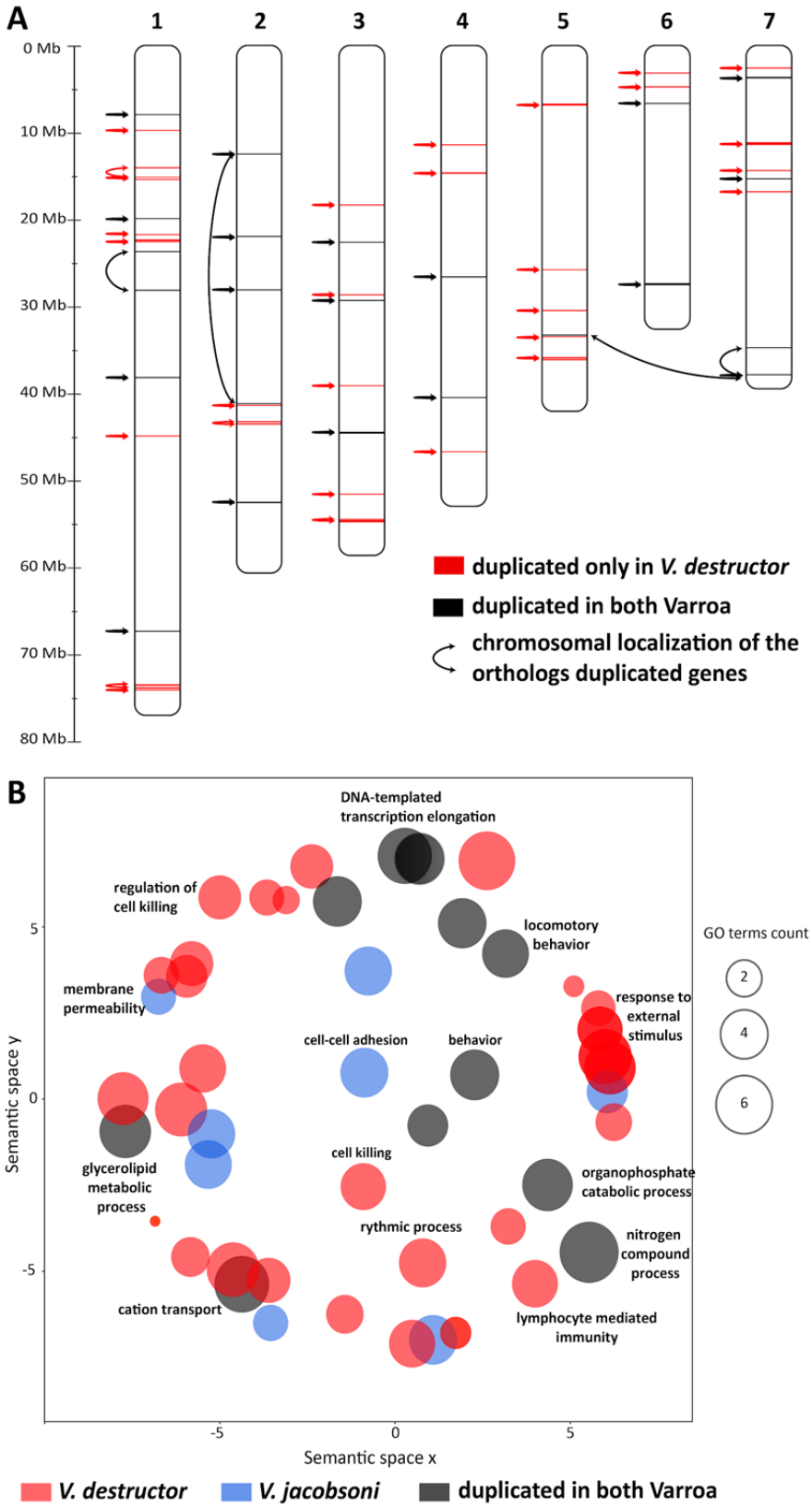
A
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60 Mb
70 Mb
80 Mb
duplicated only in V. destructor
duplicated in both Varroa
chromosomal localization of the orthologs duplicated genes
B
DNA-templated transcription elongation
regulation of cell killing
locomotory behavior
membrane permeability
response to external stimulus
cell-cell adhesion
behavior
cell killing
glycerolipid metabolic process
organophosphate catabolic process
rythmic process
nitrogen compound process
cation transport
lymphocyte mediated immunity
Semantic space y
Semantic space x
GO terms count
2
4
6
V. destructor
V. jacobsoni
duplicated in both Varroa

541 **Figure 5: Duplicated genes in *V. destructor* are found throughout the genome and are involved in**
542 **different biological pathways.** (A) Chromosomal location and similarity of duplicated genes (arrows) for
543 *V. destructor*. Different genes and chromosomal regions underwent duplication in the two species. (B)
544 Cluster analysis of significantly enriched GO terms for biological processes over-represented among
545 duplicating genes in *Varroa* mites. Not only were most of the GO terms species-specific, but they also
546 comprised non-overlapping categories of biological processes. Neither gene duplication nor selection
547 analysis suggests a substantial degree of parallel evolution in these mites.
548
549 **Chemosensory and cytochrome P450 proteins are conserved in honey bee parasites but**
550 ***Varroa* species show different selective trajectories**
551
552 **Lipid carrier proteins:** Based on the comparison of six amino acid sequences of OBPs previously
553 described from *V. destructor* legs transcripts [93], we found seven homologs (with OBP conserved
554 domain IPR036728 or OBP-like) in *V. destructor* annotated genome (Figure S4). Each OBP gene
555 found in *V. destructor* presented an ortholog in *V. jacobsoni* coding for an identical OBP
556 sequence (Supplementary Table S9). An analogous patterns of strong conservation was found
557 for eight NCP2s (IPR003172) or similar with a Ganglioside GM2 activator conserved domain
558 (IPR028996) in both sibling Varroa mites (Figure S5). None of the genes encoding for OBP and
559 NCP2 proteins showed signatures of positive selection or tandem duplication in Varroa sister
560 species.
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562

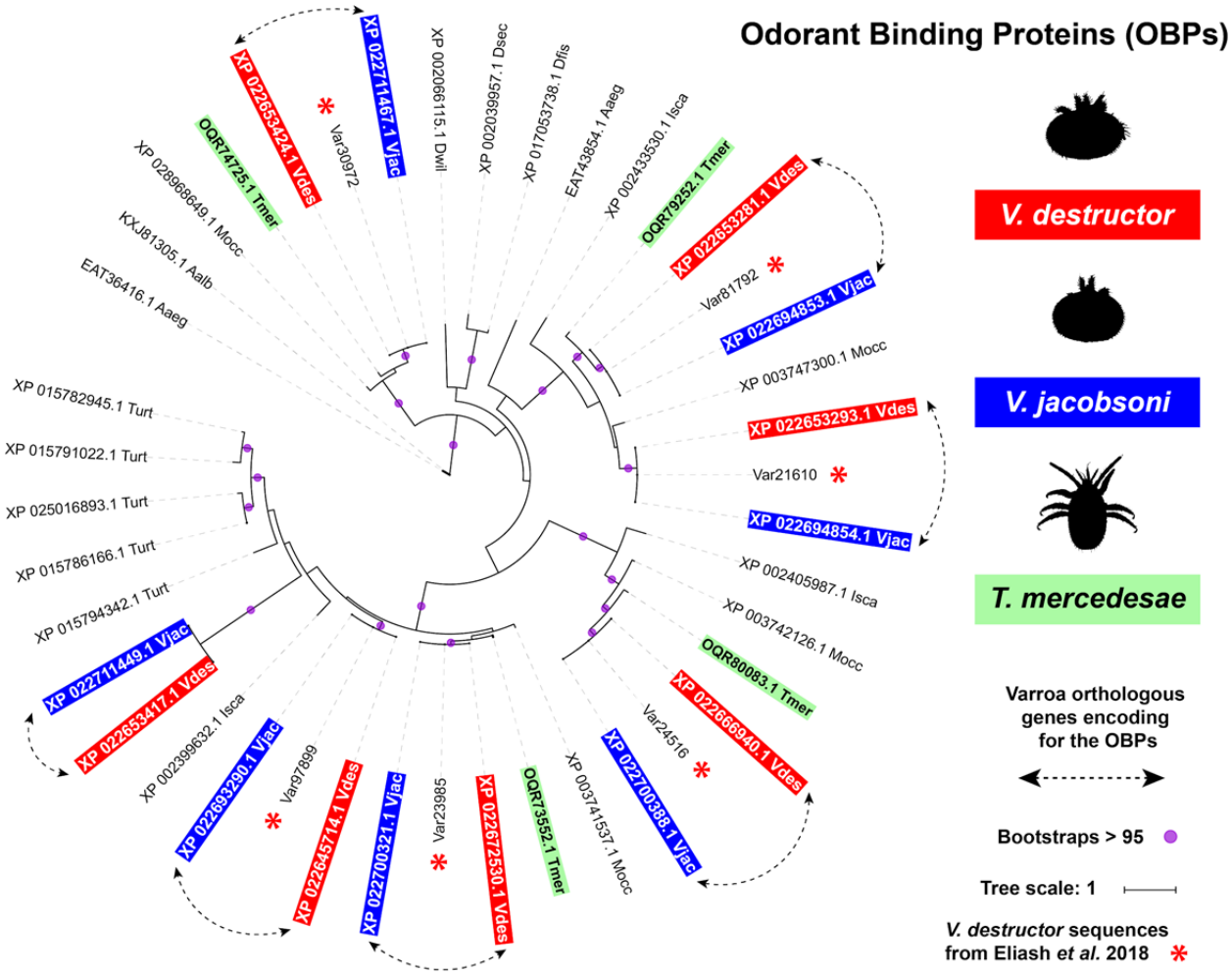

Figure S4: OBP repertoires are highly conserved between *V*arroa sibling species and other Acari. The phylogenetic tree was constructed using 42 amino acid sequences from Acari. *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca), *T. urticae* (Turt) (see list in Table S9). Additionally, sequences from mosquitos Aedes aegypti (Aaeg), *A. albopictus* (Aaeg) and fruit flies *Drosophila* (D), were downloaded from NCBI accession and aligned with MAFFT. Best fit model computed for the tree is WAG+I+G4 using IQ-TREE. Bootstrap values were estimated using an SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle.

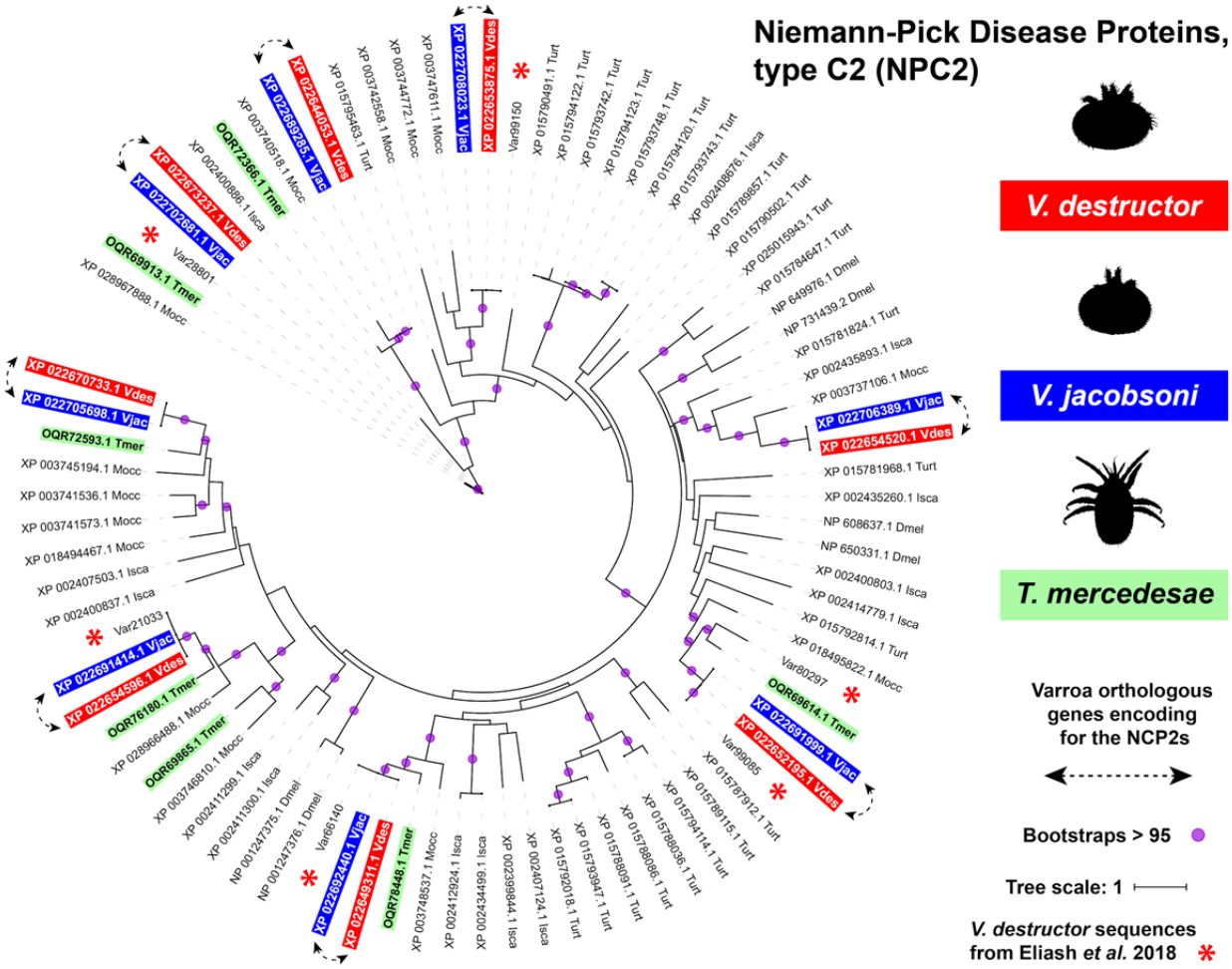

Figure S5: *V. destructor* and *V. jacobsoni* genomes showed eight NCP2 amino acid sequences mostly conserved with the other honey bee parasite *T. mercedesae*. The phylogenetic tree was constructed using 86 amino acid sequences from Acari *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca) and *T. urticae* (Turt) (see list in Table S9). Additionally, sequences from the fruit fly *Drosophila melanogaster* (Dmel), downloaded from NCBI and aligned with MAFFT. Best fit model computed for the tree is LG+R4 using IQ-TREE. Bootstrap values were estimated using an SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle.

591 **Membrane-bound receptors:** A total of four genes per Varroa genome were found to encode
592 for GR proteins presenting either the 7m_7 or Trehalose receptor conserved domains
593 (IPR013604 and IPR009318, respectively). All four GR were closely related to GRs proteins
594 found either in the other honey bee parasitic mite *T. mercedesae* or in the parasitic tick *I.*
595 *scapularis* (Figure S6). As previously reported, IGR protein profiles are the largest in *V.*
596 *destructor.* According to the IGR phylogeny, this is also the case for *V. jacobsoni* with 35
597 identified genes (mainly presenting IPR001828, IPR019594 or IPR001320 signatures) (Figure 6).
598 Among these, 19 proteins found in Varroa genomes had closest homolog in *T. mercedesae*
599 indicating high conservation of the gene family within honey bee parasites. Interestingly, 3
600 genes encoding for IGR or IGR-like proteins out of the 37 identified in *V. destructor* had no
601 orthologous in *V. jacobson*i. The opposite situation was also true but only for one gene in *V.*
602 *jacobsoni* (LOC111272277). Additionally, divergent selective trajectories were found as a
603 glutamate [NMDA] receptor subunit 1-like gene (LOC111270396) was under positive selection
604 for *V. jacobsoni* while a metabotropic glutamate receptor 3-like gene (LOC111245193) show
605 positive selection signal for *V. destructor*. Finally, despite a small number of SNMPs (with
606 conserved domain IPR002159) detected in *V. jacobsoni*, we also found that one scavenger
607 receptor class B member 1-like gene (LOC111267160) was under positive selection but none for
608 *V. destructor* (Figure S7).
609
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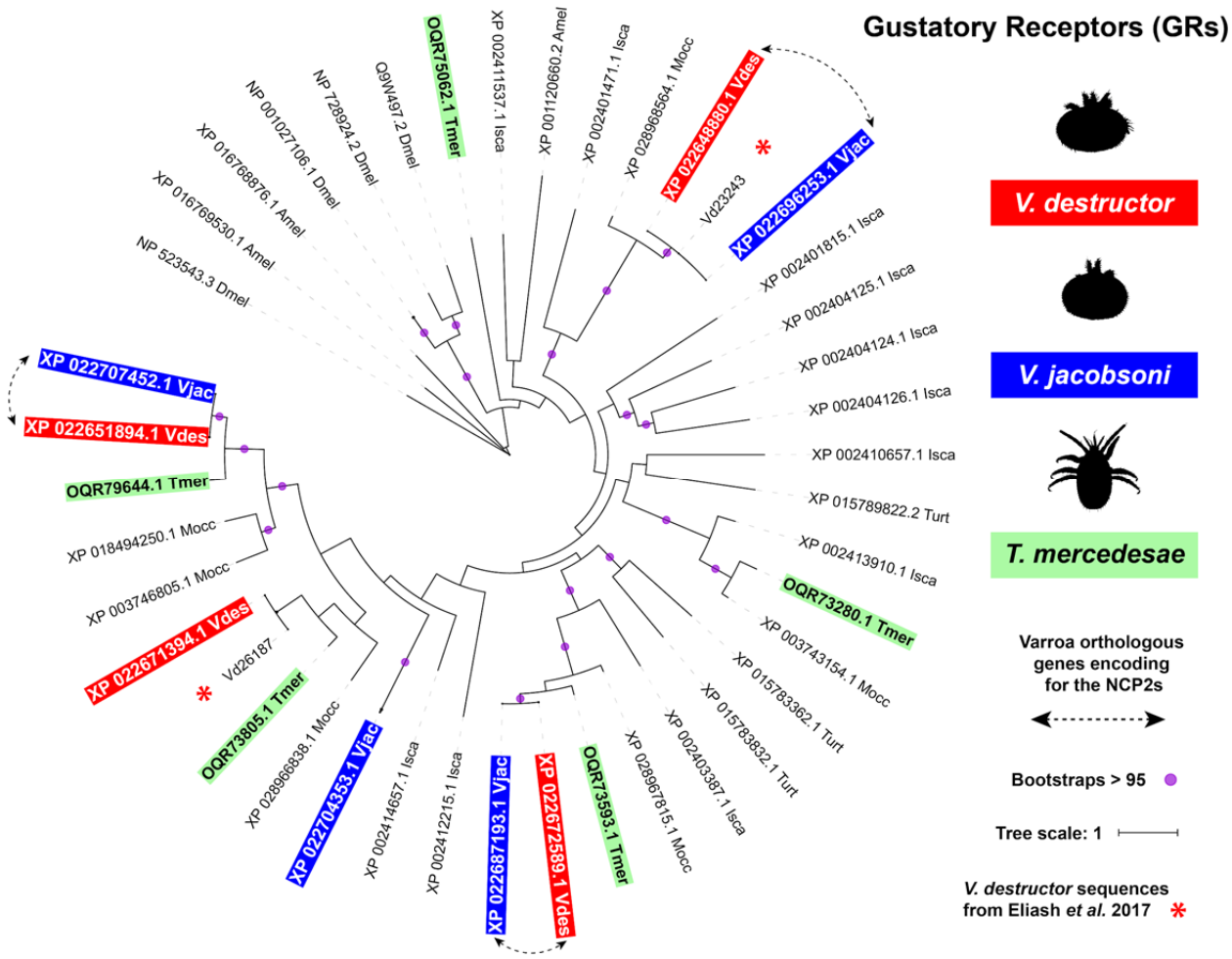

Figure S6: *V. destructor* and *V. jacobsoni* genomes presented only four gustatory receptors amino acid sequences like. The phylogenetic tree was constructed using 45 amino acid sequences from Acari *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca) and *T. urticae* (Turt) (see list in Table S9). Additionally, sequences from the fruit fly *Drosophila melanogaster* (Dmel) and the honey bee *A. mellifera (*Amel), sequences were downloaded from NCBI and aligned with MAFFT. The best-fit model computed for the tree is LG+F+R4 using IQ-TREE. Bootstrap values were estimated using an SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle.

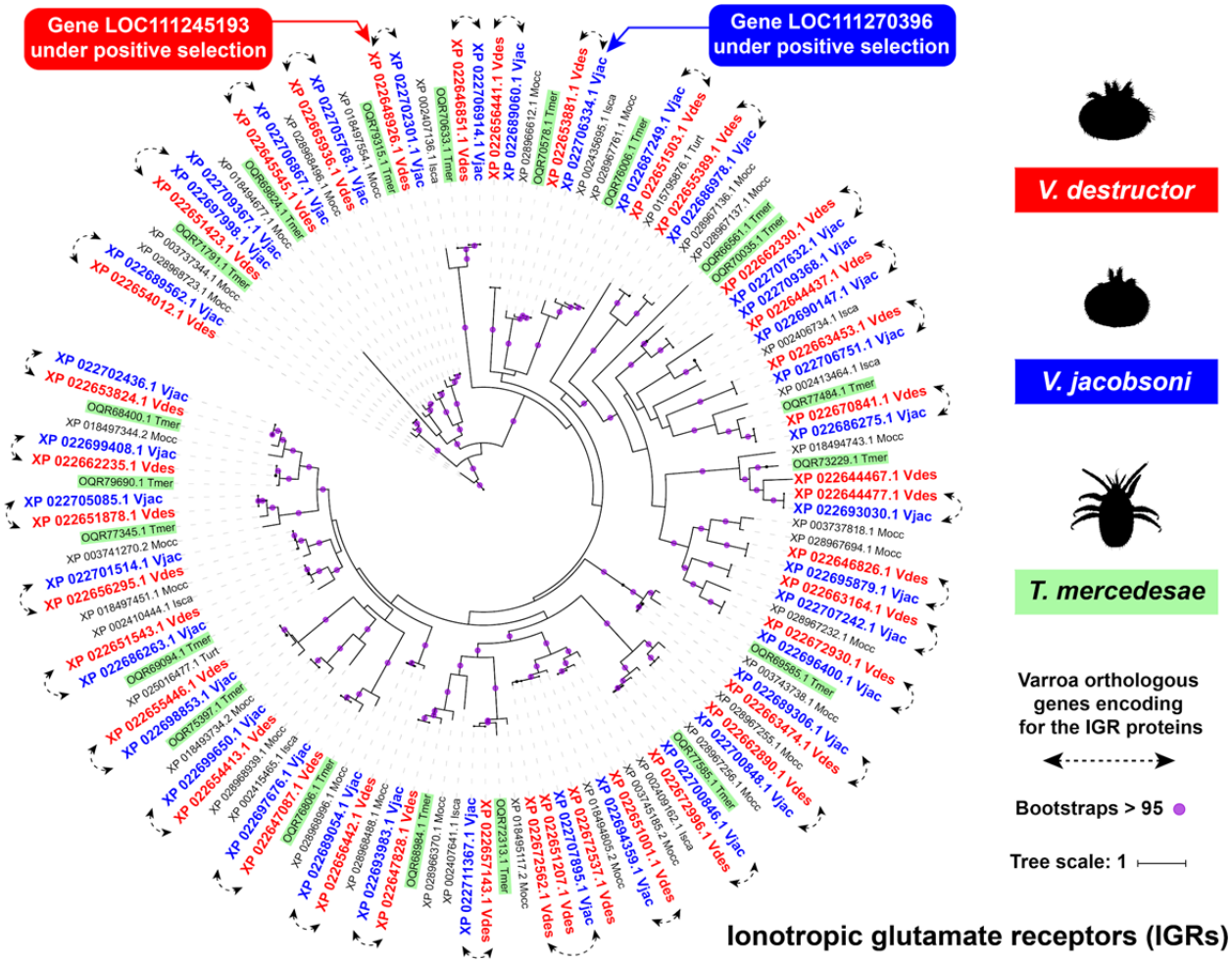

Figure 6: The largest IGR repertoire in honey bee parasites show divergent selective trajectories for host and environment chemosensing between *V. destructor* and *V. jacobsoni*. The phylogenetic tree was constructed using 153 amino acid sequences from Acari *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca) and *T. urticae* (Turt) which were aligned with MAFFT (see list in Table S9). Best fit model computed for the tree is VT+R5 using IQ-TREE. Bootstrap values were estimated using an SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle.

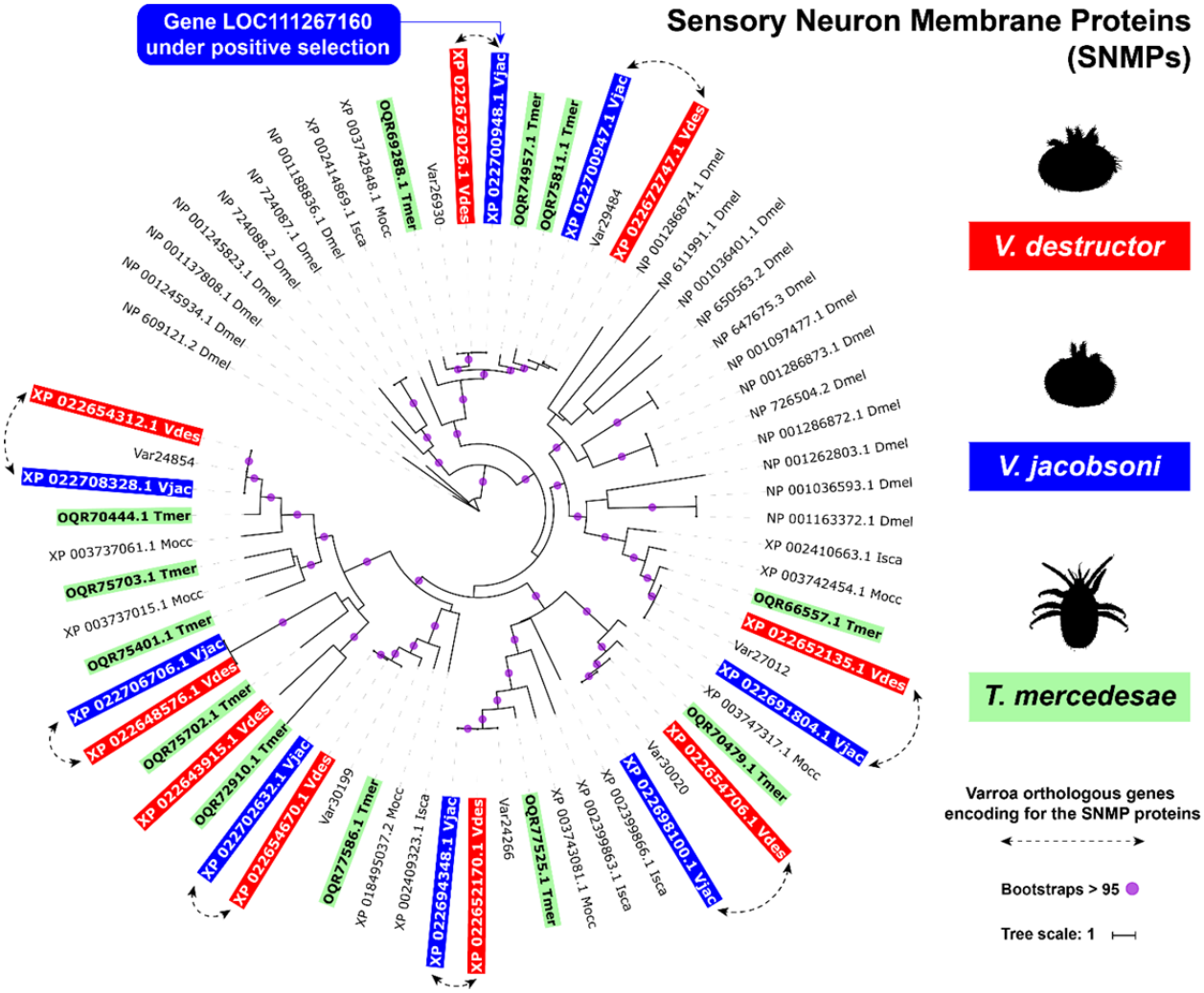

Figure S7: Sensory neuron membrane protein repertoires are conserved among honey bee parasites but sibling Varroa species show signs of differential selective regime. The phylogenetic tree was constructed using 67 amino acid sequences from Acari *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca) (see list in Table S9). Additionally, sequences from the fruit fly *Drosophila melanogaster* (Dmel), were implemented with NCBI accession and aligned with MAFFT. Best fit model computed for the tree is LG+R5 using IQ-TREE. Bootstrap values were estimated using an SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle.

Ecdysteroids and Halloween genes: All CYP enzymes sequences from *V. destructor* and *V. jacobsoni* annotation were extracted by filtering gene description list containing "cytochrome p450" or exhibiting the associated conserved domain cl12078 or pfam00067. A total of 36 and 38 CYP (one isoform per gene) were aligned together with the seven Halloween genes reported from *D. melanogaster* and *A. aegypti* and other CYPs from Acari. Previously, only some members of this pathway, namely orthologues of *spook*, *shade* and *disembodied* were found from a genomic sequence generated by 454 pyrosequencing [44]. Homologs proteins of CYP302A1 and CYP314A1 (~32% and ~28% sequence identity to *disembodied* and *shade* of *D. melanogaster,* respectively) were found for both Varroa sibling species but surprisingly no homolog *spook* (CYP307A1) was found for *V. jacobsoni*, though there was one in *V. destructor* (Figure 7). Phylogenetic relationships among Varroa mites, *T. urticae* and *M. occidentalis* and *D. melanogaster* CYP315A1 sequences (~36% identity with the fruit fly) suggest that both *V. destructor* and *V. jacobsoni* Varroa mites possess *shadow* homologs. Likely, *shroud* and *phantom* are also presents in both Varroa species but not *neverland*. In the CYP family, we detected three genes under positive selection only in *V. jacobsoni*, including *shadow*-like (LOC111271433) and disembodied (LOC111265704) (Figure 7 and Table S9).

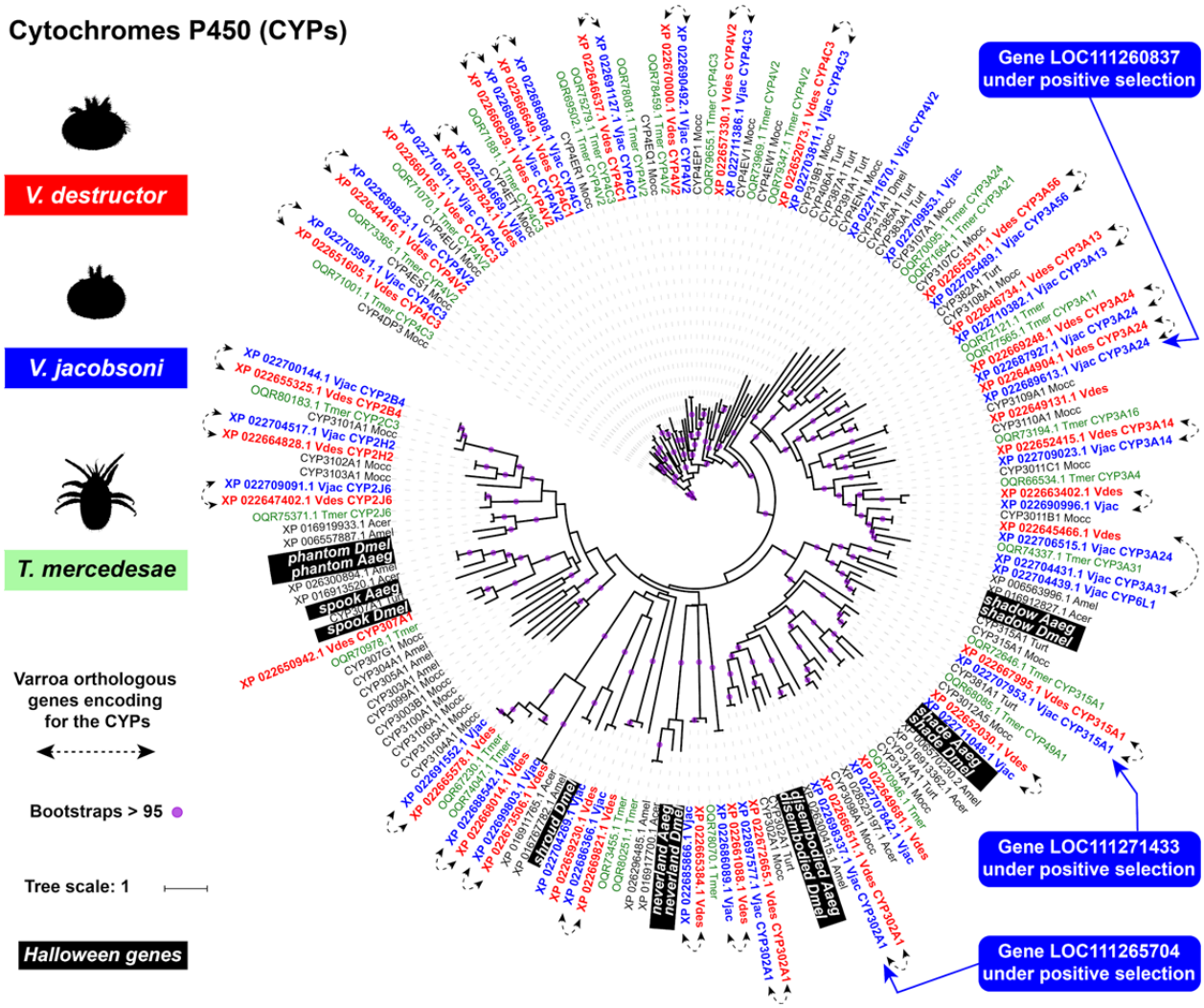

Figure 7: CYP repertoire in Varroa mites is broader than expected with two functionaly important Halloween genes *shade* and *disembodied* under positive selection in *V. jacobsoni*. The phylogenetic tree was constructed using 174 amino acid sequences from Acari *V. destructor* (Vdes = genome and Var = protein sequences from Eliash et al. 2018), *V. jacobsoni* (Vjac), *T. mercedesae* (Tmer), *M. occidentalis* (Mocc), *I. scapularis* (Isca) and *T. urticae* (Turt). Additionally, sequences from the fruit fly *Drosophila melanogaster* (Dmel), mosquito *Aedes aegypti* (Aaeg) and the honey bee *A. mellifera (*Amel) and *A. cerana* (Acer) were downloaded form NCBI and aligned with MAFFT. Best fit model computed for the tree is LG+R5 using IQ-TREE. Bootstrap values were estimated using a SH-like aLRT with 1000 and bootstraps over 95% are shown by a purple circle. *V. destructor* was previously believed to have only *disembodied* (CYP302a1)*, shade* (CYP314a1) and *spook* (CYP307a1) homologs of the seven Halloween genes (background in black)[44].

Other genes possibly related to nutrition and detoxification: Some annotated genes detected under positive selection or undergoing tandem duplication in *Varroa* have been 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). On *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 physical properties of the mite cuticle, which in addition to providing protection may constitute the first barrier to reduce the penetration of external chemical agents [104]. 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. These were not detected for *V. jacobsoni* (Table 2). Both positive gene selection and duplication patterns in the *V. destructor* and *V. jacobsoni* genomes and related GO terms showed that the two sister species have undergone dissimilar evolutionary trajectories.

Table 2: Genes undergoing positive selection or duplication, that have been implicated in tolerance to external stressors and stimuli. Research on Acari and *V. destructor*, in particular, has identified a large number of genes involved in stress and detoxification (possibly leading to acaricide resistance), nutrition, reproduction and chemosensing in interaction with the host. Intriguingly, many of these genes appear to be under ancestral positive selection, as *V. jacobsoni* has not been extensively targeted by acaricides on *A. mellifera*, and most of the evolutionary history captured by positive selection analyses occurred in *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)**	
Stress from in-hive temperature	**Annotated gene under positive selection**	**Annotated gene undergoing duplication**	**Annotated gene under positive selection**	**Annotated gene undergoing duplication**
Heat Shock 70 Kda protein-like [105]		6		

Nutrition				
Guanine metabolism [106]			1	
Detoxification				
Glutathione-S-transferase [107]			1	
Sodium channel related [108]	4		2	
Transmembrane proteins	3		4	2
G-protein coupled receptors	1			
Esterases [109]	2			
GABA-gated ion channels [110]	1			
ATP-binding cassette gene family [111]	1		2	2
Molting and reproduction				
Ecdysteroids pathway with cytochrome P450 [44]			3	
Cuticular protein	2	4		1
Nutrition and reproduction				
Fatty acid metabolism [97,112,113]	3			
Chemosensory receptors				
Sensory neuron membrane protein			1	
Ionotropic glutamate receptor	1		1	

718 **Discussion**

719

720 In this study, we developed high-quality *de novo* reference genomes and annotations for
721 economically important honey bee parasites *V. destructor* and *V. jacobsoni*. Because *Varroa*
722 mites are, to a large extent, responsible for the global honey bee crisis, and research efforts
723 worldwide target these parasites, these genomic resources respond to a pressing need [36].
724 Improved genome assembly and annotation with transcriptomic data from different life stages
725 produced the most comprehensive gene catalogue to date, as reflected by our discovery of
726 previously missing Halloween genes homologs [44]. The supposed absence of such genes has led
727 to speculation control measures development or host counter adaptation could target this
728 pathway[45]. Our analysis also revealed that the two *Varroa* species share 99.7% sequence
729 identity across the nuclear genome. Yet, after the two species have split, they have gone on
730 largely dissimilar evolutionary trajectories, as evidenced by different patterns of positive
731 selection and gene duplication. In particular, signals of positive selection were found in
732 functionally important genes involved in chemosensing, molting, and reproduction. Thus, it
733 seems likely that the two species underwent different patterns of adaptation while continuing
734 to exploit the same host, the eastern honey bee (*A. cerana*). Differences between the two
735 species most likely result from local adaptation to host geographic subspecies, the environment
736 or their interaction. Though not enough data exist to distinguish between these alternatives,
737 below we summarize what is already known and outline promising avenues for investigation.

738

739 Inferring what constitutes the ancestral ecological niche of the two mites is key to
740 understanding what selective pressures they have faced and how they responded to them. *V.*
741 *destructor* and *V. jacobsoni* are very similar morphologically, and until 20 years ago they were
742 mistakenly considered the same species [28]. Their host specificity overlaps as they both
743 parasitize the eastern honey bee *A. cerana* throughout Asia. The only exception reported for
744 the Indonesian *A. nigrocincta* infested by *V. jacobsoni*, though possibly as a result of a recent
745 host switch from *A. cerana* [28,114]. The most recent common ancestor of *V. destructor* and *V.*
746 *jacobsoni* might have also been a parasite of the *A. cerana* lineage. This hypothesis is supported
747 by the host range of *V. underwoodi*, another closely related mite that also parasitizes *A. cerana*
748 in addition to *A. nigrocincta* and *A. nulensis* [115,116]. With no known varroid (Mesostigmata)
749 fossils, no information exists about speciation timing in *Varroa* mites. Based on the *Cox1* gene,
750 a recognized molecular clock for insects with a divergence rate of 2.3% per Myr [117], the
751 divergence between *V. destructor* and *V. jacobsoni* of 5.9% suggests that the two species split
752 ~2-3 Myr ago. This is after the split between *A. cerana* and *A. mellifera* which occurred 6-9 Myr

753 ago based on *Cox1* and *ND2* [118,119] to 17-19 Myr using a combination of mitochondrial,
754 ribosomal and nuclear *loci* [120]. Thus, host ranges and molecular estimates suggest that most
755 *Varroa* evolution occurred on ancestors of *A. cerana*.
756
757 The major ecological difference between these mites is their parapatric distribution
758 (Figure 1). Whether parapatry resulted from their respective biogeographic history, adaptation
759 to different subspecies of *A. cerana*, or is shaped by competition is not known. In fact, even the
760 extent of the geographical overlap between the mite distributions is uncertain, since few
761 surveys actually used molecular markers for identification and most have focused on
762 associations with *A. mellifera* [28,72,116,121]. Yet, parapatry is clearly not due to the inability of the
763 mites to complete their phoretic life stage outside the hive, as host-switched V*. destructor*
764 successfully expanded its range following the range extension of *A. mellifera*. While allopatry
765 could result from adaptation to local hosts/conditions or competitive exclusion, both mites
766 have been reported from the same Thai apiary on *A. cerana* [46,48]. Unfortunately, there are not
767 enough data on *A. cerana* infestation under natural conditions to determine the likelihood that
768 the two *Varroa* species interact and compete. One survey in southern Thailand found 98%
769 parasitism by *V. jacobsoni* under natural conditions, albeit at variable levels of infestation [122],
770 suggesting that encounters between the two species are indeed likely. If the divergent
771 evolutionary history of the two *Varroa* species resulted from competition, we predict that long-
772 term coexistence within a hive of *A. cerana* should be unlikely, a hypothesis that we hope will
773 be tested in the future in the zone of sympatry.
774
775 In addition, or as an alternative to interspecific competition, it is possible that the
776 divergence between *V. destructor* and *V. jacobsoni* was driven by local adaptation to different
777 *A. cerana* populations. For instance, *V. destructor* was found to infect the "Northern *cerana*",
778 "Himalayan *cerana*" and the "Indo-Chinese *cerana*" morpho-clusters identified by [47]
779 whereas *V. jacobsoni* was reported on the "Indo-Chinese *cerana*" and the "Indo-Malayan
780 *cerana*". These *A. cerana* populations differ in their nesting behaviour, such as the number of
781 combs or even in their body size [80]. In China alone, *A. cerana* populations have been shown to
782 be as genetically divergent for more than 300,000 to 500,000 years as recognized subspecies of
783 *A. mellifera* [123]. In addition to genetic differences between the hosts, the climactic
784 environments across Asia are highly variable and could result in local adaptation, though, as
785 mentioned earlier, this did not prevent range expansions after switching hosts (Figure 1).
786 Consequently, the dissimilar selective pressures observed in the mites may have resulted from
787 selection due to different host genotypes.

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 [30]. 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 replace each other locally is unknown. Our data suggest that if the parasites are able to tolerate each other within *A. mellifera* colonies, given their different suites of molecular adaptations, they may in fact impose a greater cost than either 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 genomes show signs of selection on a range of genes previously found to be involved in stress and pesticide resistance (Table 2). Since *V. destructor* has evolved resistance to acaricides, it cannot be ruled out that positive selection resulted from recent acaricide pressures rather than co-evolution with their host. By contrast, acaricides are not yet widely used on *V. jacobsoni*, though the number of genes under selection is similar, suggesting that most selection is ancient. In the case of *V. jacobsoni*, and possibly generally, these genes have the potential to become co-opted for pesticide resistance. 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 [124]. Similarly, the two-spotted mite (*Tetranychus urticae*) appears to have co-opted existing detoxification machinery in a selective response to miticides [125]. 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 [126].

Conclusion

Despite the economic importance of brood parasitic mites in *A. mellifera*, it is clear that various pressing research needs still exist concerning their biology, cryptic diversity in the native range, possible geographic ranges of host-switched species, and how co-infestations may proceed. These new reference genomes will allow researchers to pursue such investigations, develop better tools for *Varroa* control, and to better understand the invasion and evolution of *Varroa* on *A. mellifera*. In particular, we hope that these new resources will greatly benefit

823 future studies to track the population genetic changes and demography of *V. destructor*'s
824 worldwide spread, which has been difficult due to the low polymorphism of previously available
825 markers [121]. More *Varroa spp.* are continuously testing new host [73,75] and may remain
826 undetected. Finally, the recent boom in -omics methods for the study of honey bee parasites
827 and diseases underlines the need to improve our knowledge of genome structure and
828 organization to investigate the genetic basis of newly reported acaricide resistance, virulence
829 level or even new host tolerance. Such knowledge combined with progress in novel
830 manipulation tools, such as RNAi [107,127], and more experimental tools like gene drive [128], may
831 provide ways of specifically controlling mite pests without harming honey bees or other insects.
832
833
834

Supplementary data

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

Figure S2: Vdes3.0 clusters with the worldwide invasive V. destructor K1-1/K1-2 haplogroup. Maximum likelihood tree was build on 5000 bootstraps using concatenate sequences of *Cox1, Cox3, Atp6* and *Cytb* partial genes.

Figure S3: UpSet plot of orthologous genes detected under positive selection detailing the shared number per species.

Figure S4: Treemap plots (REVIGO) analysis for the summary of GO terms in biological processes for gene detected under positive selection when comparing Varroa species.

Supplementary table 1: Summary output of orthologs genes and correspondence among the six species included in the phylogeny from ORTHONOME pipeline

Supplementary table 2: GO terms enrichment for ortholog clusters before species split between Varroa mites and with four other Acari

Supplementary table 3: Genomic details of the genes detected under positive either on *V. destructor, V. jacobsoni* or Varroa ancestral (before species split) branch

Supplementary table 04: GO terms enrichment analysis of genes detected under positive selection for *V. destructor, V. jacobsoni* and Varroa ancestral branch (before species split)

Supplementary table 05: Gene duplicated in tandem different between *V. destructo*r and *V. jacobsoni* from the MSOAR2 pipeline

Supplementary table 06: Gene duplicated in tandem shared by Varroa mites and Tropilaelaps

Supplementary table 07: GO terms enrichment analysis of duplicated in tandem genes for *V. destructor* and *V. jacobsoni*

870 **Supplementary table 08:** Gene set enrichment analysis of genes duplicated shared by Varroa
871 mites
872
873 **Supplementary table 09:** Details of the proteins and related genes used for phylogenetic
874 analysis and comparison of chemosensory proteins
875
876

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889

890 Data accessibility

891
892 *V. destructor* (Vdes_3.0) and *V. jacobsoni* (Vjacob_1.0) assembled reference genomes and
893 annotation are available on NCBI database respectively with the accession number
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895 Sequence Read Archive (SRA) under the Bioprojects PRJDB6279 and PRJNA391052. Online
896 supplemental codes and map are available with an R markdown hosted from on
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898

899 Author contributions

900 MAT collected samples for sequencing, carried out analyses, interpreted the data and wrote the
901 manuscript;
902 RVR carried out analyses and wrote the manuscript;
903 JMKR collected samples for sequencing;
904 MLG carried out the assembly and quality analyses;

905 SS carried out the computational analyses for Hi-C genome assembly;
906 IL carried out the experimental protocols for Hi-C genome assembly;
907 AKC conceived and designed experiments;
908 JDE conceived and designed experiments;
909 ASM conceived and designed experiments, carried out the assembly and analyses, interpreted the data
910 and wrote the manuscript.
911
912

913 Ethics approval and consent to participate

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916 Consent for publication

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920 IL and STS are employees and shareholders of Phase Genomics, a company commercializing
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922

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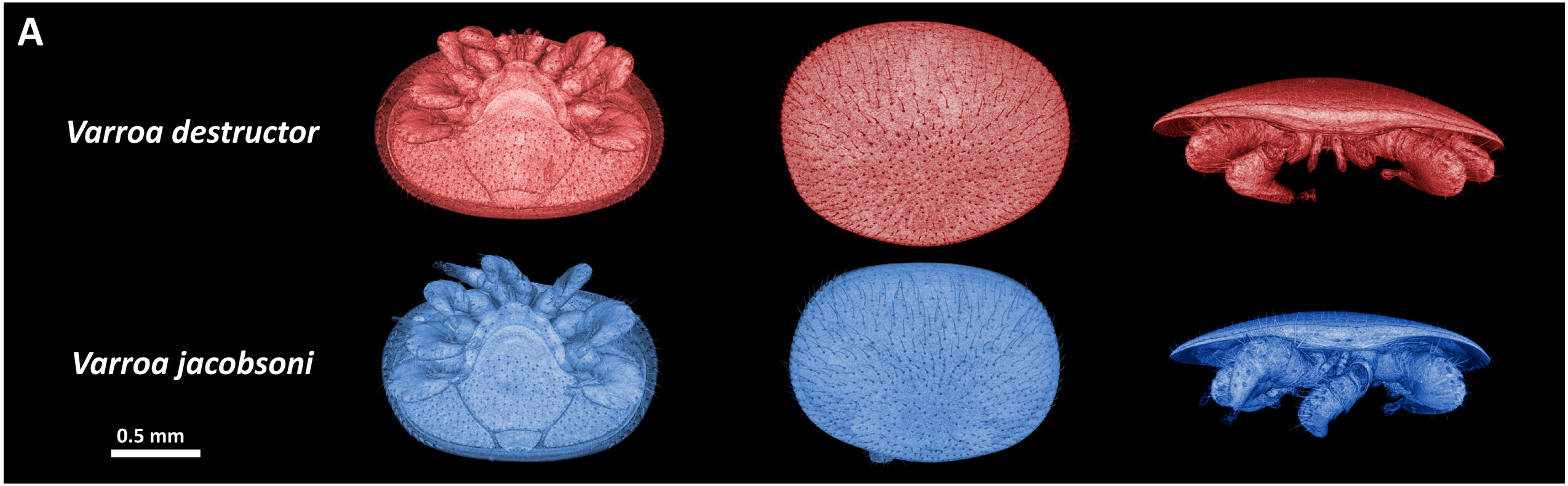
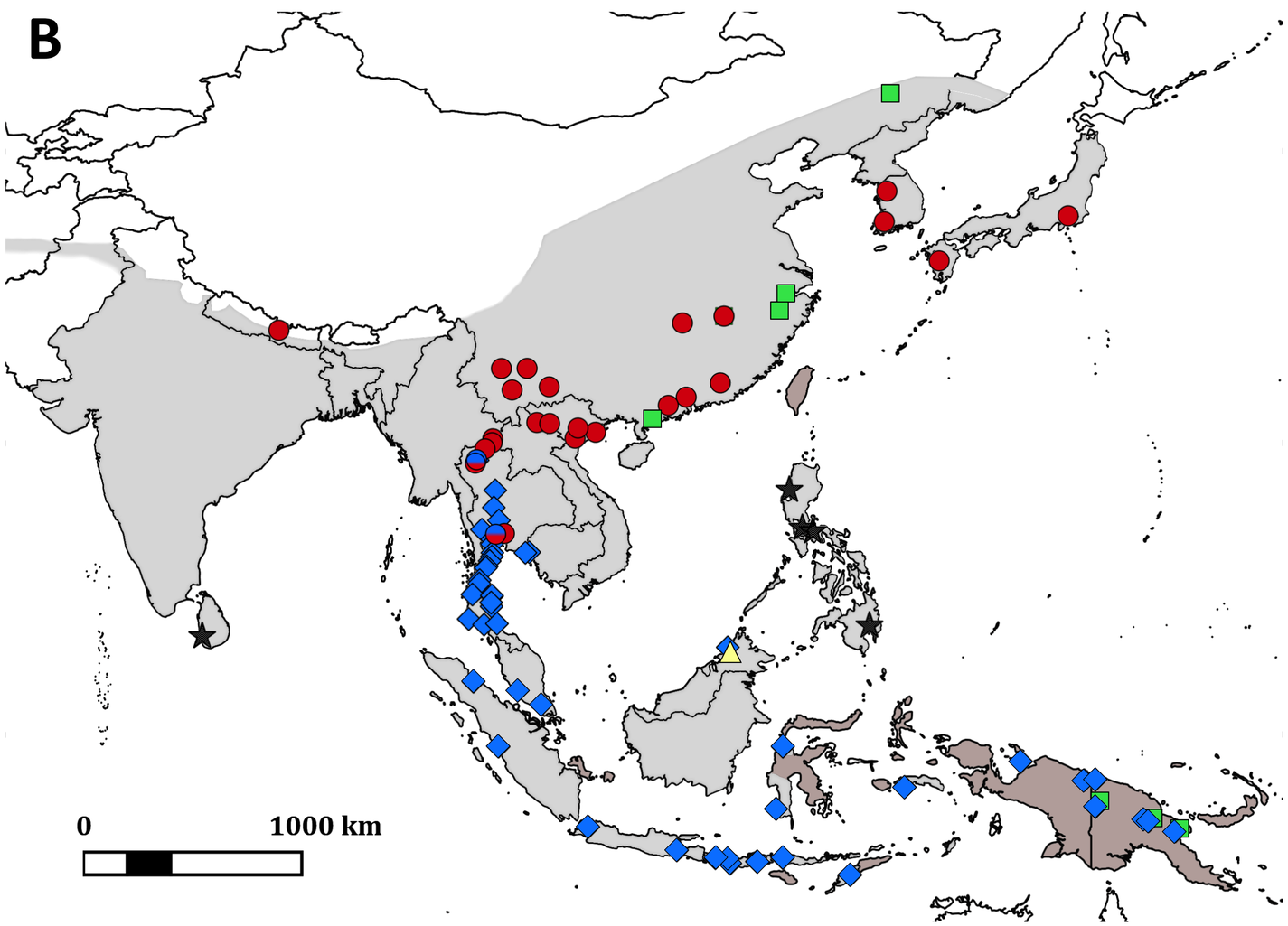
1215





Original host
A. cerana

native range

introduced range

New host
A. mellifera

Varroa cryptic species complex infecting *A. cerana* colonies

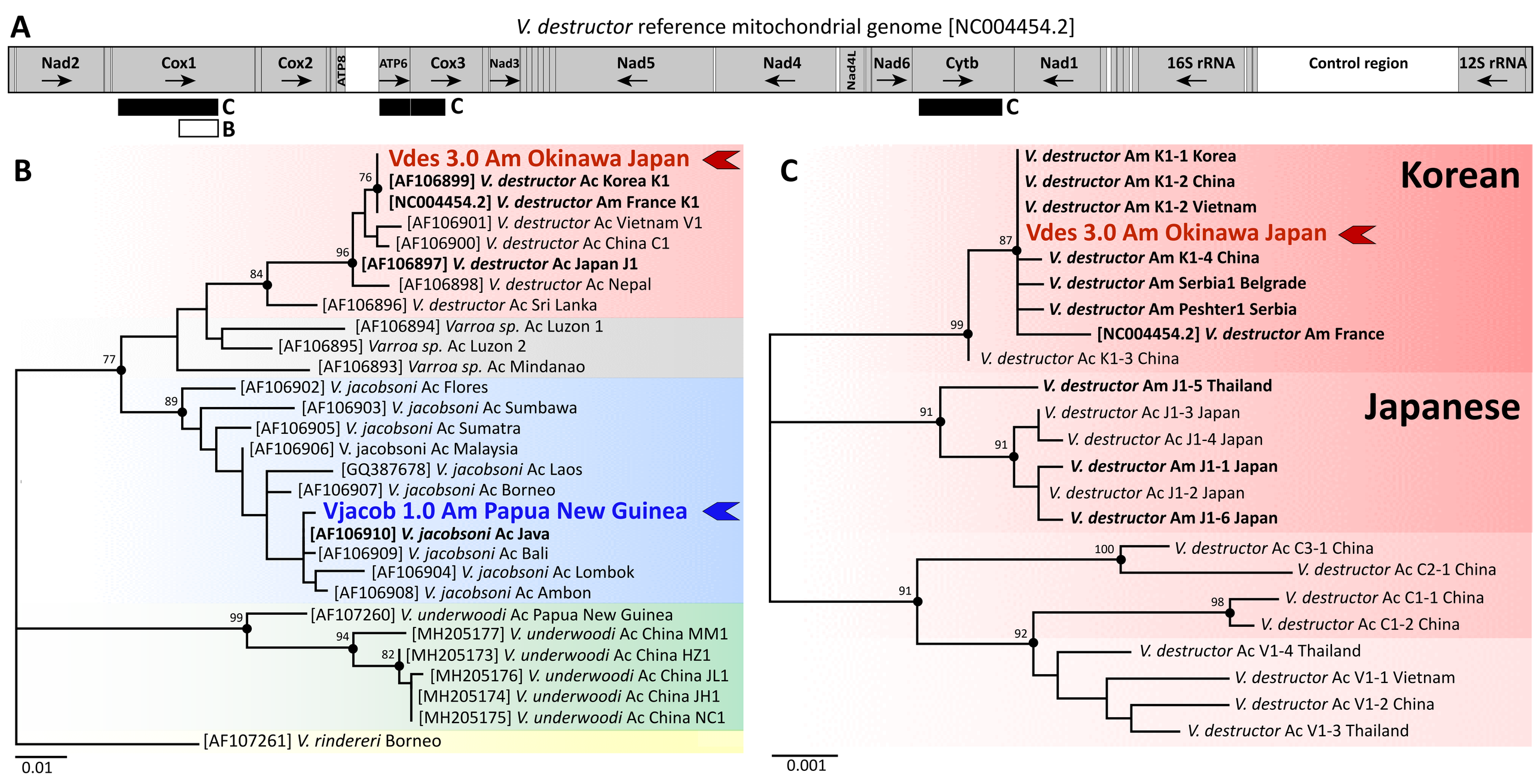
- *V. destructor*
- *V. destructor / V. jacobsoni* coexisting on the same site
- *V. jacobsoni*

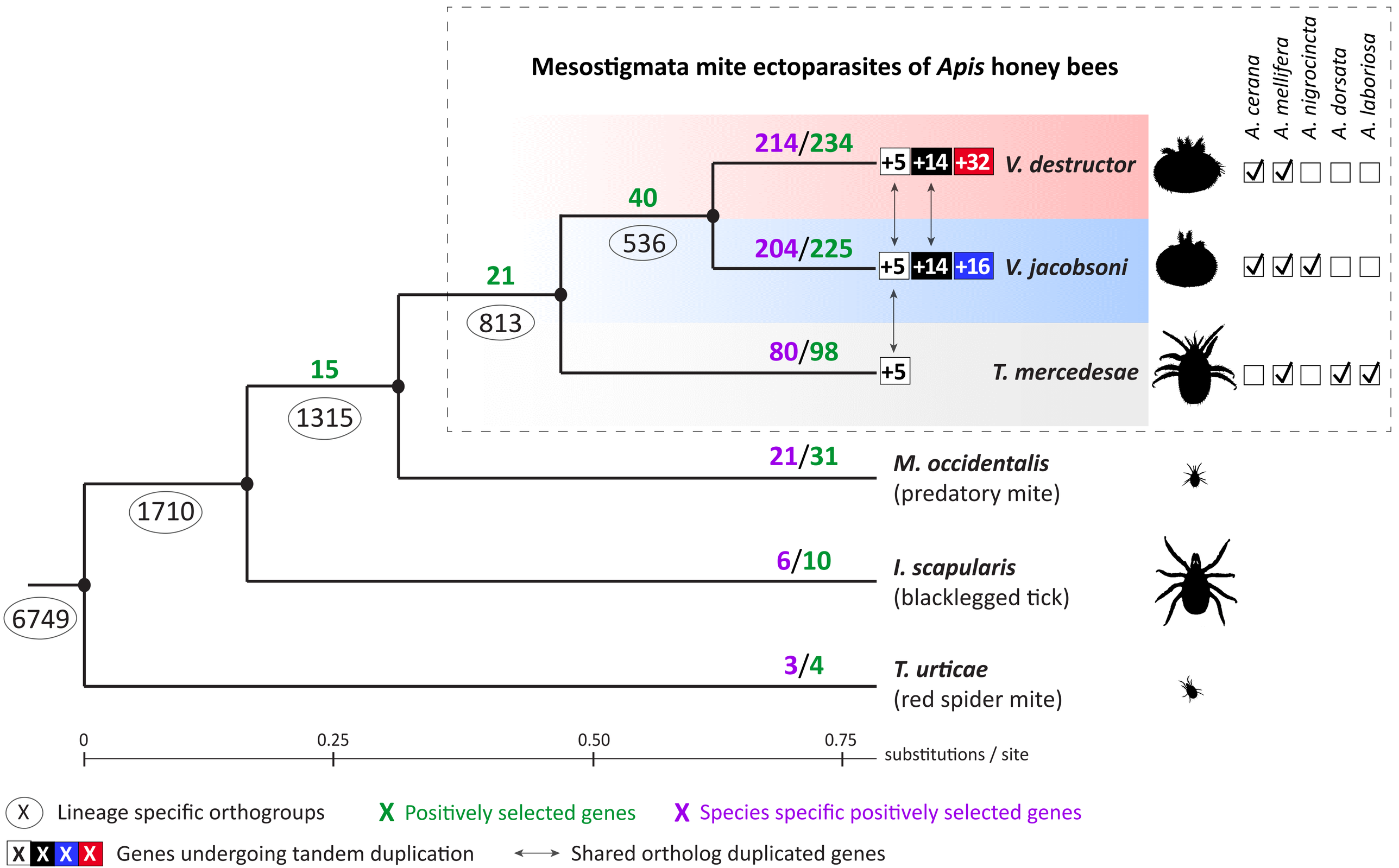
Other Varroa species known to infect *A. cerana*

- *V. underwoodi*
- *Varroa sp.* (unresolved)

Varroa species only infecting *A. koschevnikovi*

- *V. rindereri*


A
V. destructor reference mitochondrial genome [NC004454.2]
Nad2
Cox1
Cox2
ATP8
ATP6
Cox3
Nad3
Nad5
Nad4
Nad4L
Nad6
Cytb
Nad1
16S rRNA
Control region
12S rRNA
C
B
C
C
B
Vdes 3.0 Am Okinawa Japan
[AF106899] V. destructor Ac Korea K1
[NC004454.2] V. destructor Am France K1
[AF106901] V. destructor Ac Vietnam V1
[AF106900] V. destructor Ac China C1
[AF106897] V. destructor Ac Japan J1
[AF106898] V. destructor Ac Nepal
[AF106896] V. destructor Ac Sri Lanka
[AF106894] Varroa sp. Ac Luzon 1
[AF106895] Varroa sp. Ac Luzon 2
[AF106893] Varroa sp. Ac Mindanao
[AF106902] V. jacobsoni Ac Flores
[AF106903] V. jacobsoni Ac Sumbawa
[AF106905] V. jacobsoni Ac Sumatra
[AF106906] V. jacobsoni Ac Malaysia
[GQ387678] V. jacobsoni Ac Laos
[AF106907] V. jacobsoni Ac Borneo
Vjacob 1.0 Am Papua New Guinea
[AF106910] V. jacobsoni Ac Java
[AF106909] V. jacobsoni Ac Bali
[AF106904] V. jacobsoni Ac Lombok
[AF106908] V. jacobsoni Ac Ambon
[AF107260] V. underwoodi Ac Papua New Guinea
[MH205177] V. underwoodi Ac China MM1
[MH205173] V. underwoodi Ac China HZ1
[MH205176] V. underwoodi Ac China JL1
[MH205174] V. underwoodi Ac China JH1
[MH205175] V. underwoodi Ac China NC1
[AF107261] V. rindereri Borneo
76
96
84
77
89
99
94
82
0.01
C
Korean
V. destructor Am K1-1 Korea
V. destructor Am K1-2 China
V. destructor Am K1-2 Vietnam
Vdes 3.0 Am Okinawa Japan
V. destructor Am K1-4 China
V. destructor Am Serbia1 Belgrade
V. destructor Am Peshter1 Serbia
[NC004454.2] V. destructor Am France
V. destructor Ac K1-3 China
Japanese
V. destructor Am J1-5 Thailand
V. destructor Ac J1-3 Japan
V. destructor Ac J1-4 Japan
V. destructor Am J1-1 Japan
V. destructor Ac J1-2 Japan
V. destructor Am J1-6 Japan
V. destructor Ac C3-1 China
V. destructor Ac C2-1 China
V. destructor Ac C1-1 China
V. destructor Ac C1-2 China
V. destructor Ac V1-4 Thailand
V. destructor Ac V1-1 Vietnam
V. destructor Ac V1-2 China
V. destructor Ac V1-3 Thailand
87
99
91
91
100
91
98
92
0.001


Mesostigmata mite ectoparasites of Apis honey bees
A. cerana
A. mellifera
A. nigrocincta
A. dorsata
A. laboriosa
214/234
+5
+14
+32
V. destructor
40
536
204/225
+5
+14
+16
V. jacobsoni
21
813
80/98
+5
T. mercedesae
15
1315
21/31
M. occidentalis
(predatory mite)
1710
6/10
I. scapularis
(blacklegged tick)
6749
3/4
T. urticae
(red spider mite)
0
0.25
0.50
0.75
substitutions / site
X Lineage specific orthogroups
X Positively selected genes
X Species specific positively selected genes
X X X X Genes undergoing tandem duplication
Shared ortholog duplicated genes

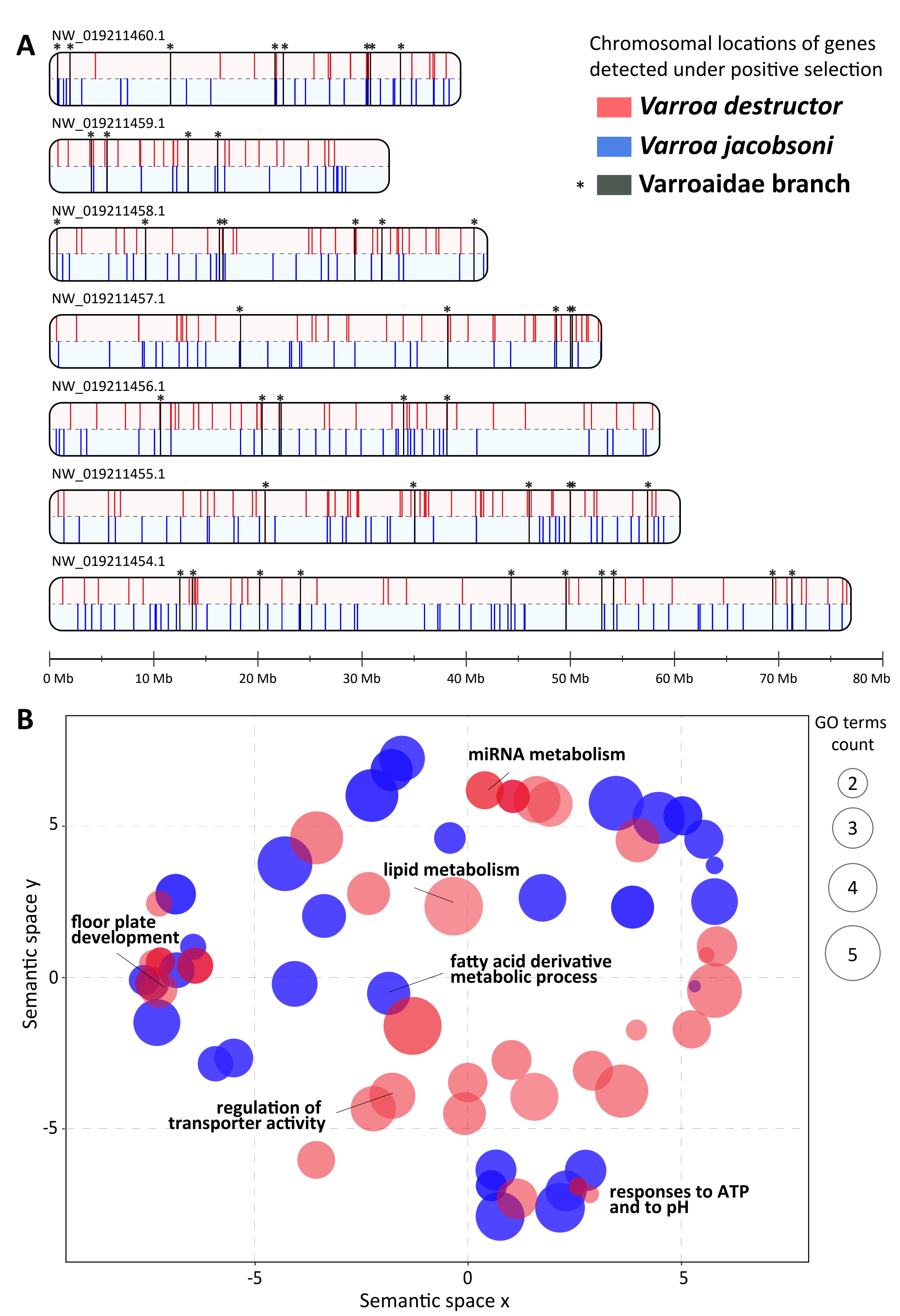
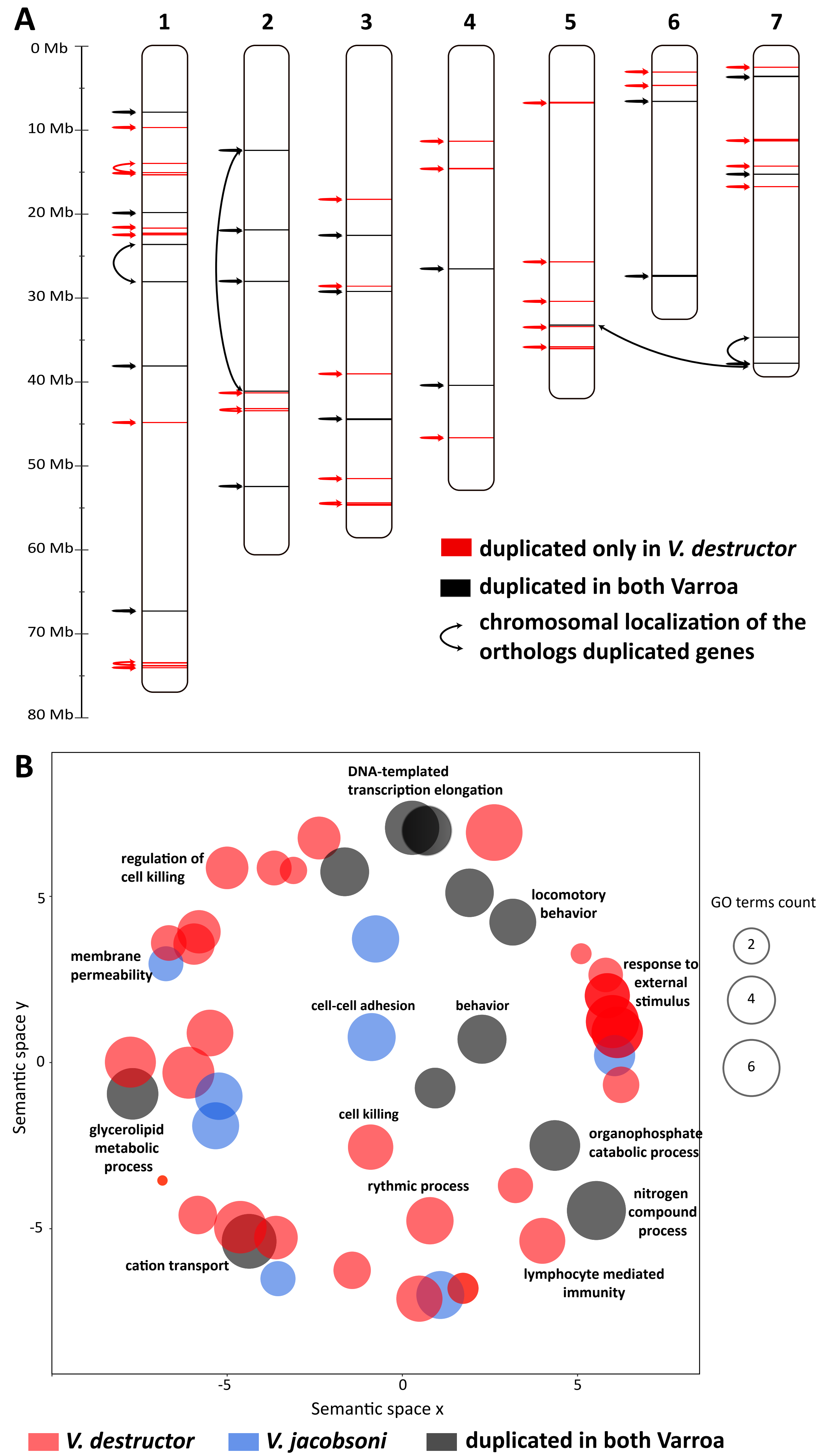

A
Chromosomal locations of genes detected under positive selection
Varroa destructor
Varroa jacobsoni
* Varroaidae branch
NW_019211460.1
NW_019211459.1
NW_019211458.1
NW_019211457.1
NW_019211456.1
NW_019211455.1
NW_019211454.1
0 Mb
10 Mb
20 Mb
30 Mb
40 Mb
50 Mb
60 Mb
70 Mb
80 Mb
B
GO terms count
2
3
4
5
miRNA metabolism
lipid metabolism
floor plate development
fatty acid derivative metabolic process
regulation of transporter activity
responses to ATP and to pH
Semantic space y
Semantic space x
5
0
-5
-5
0
5

Figure 5.


A
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4
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6
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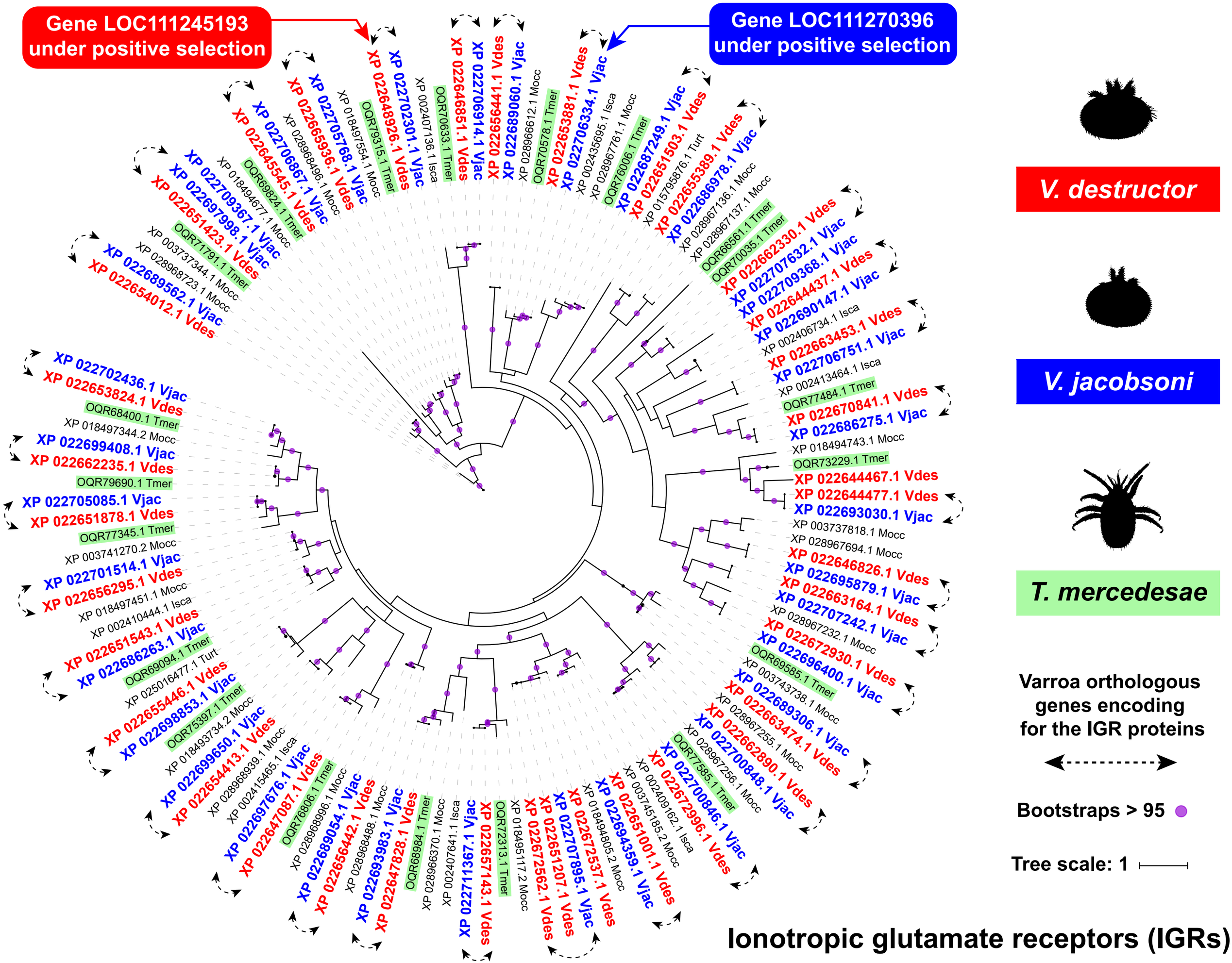
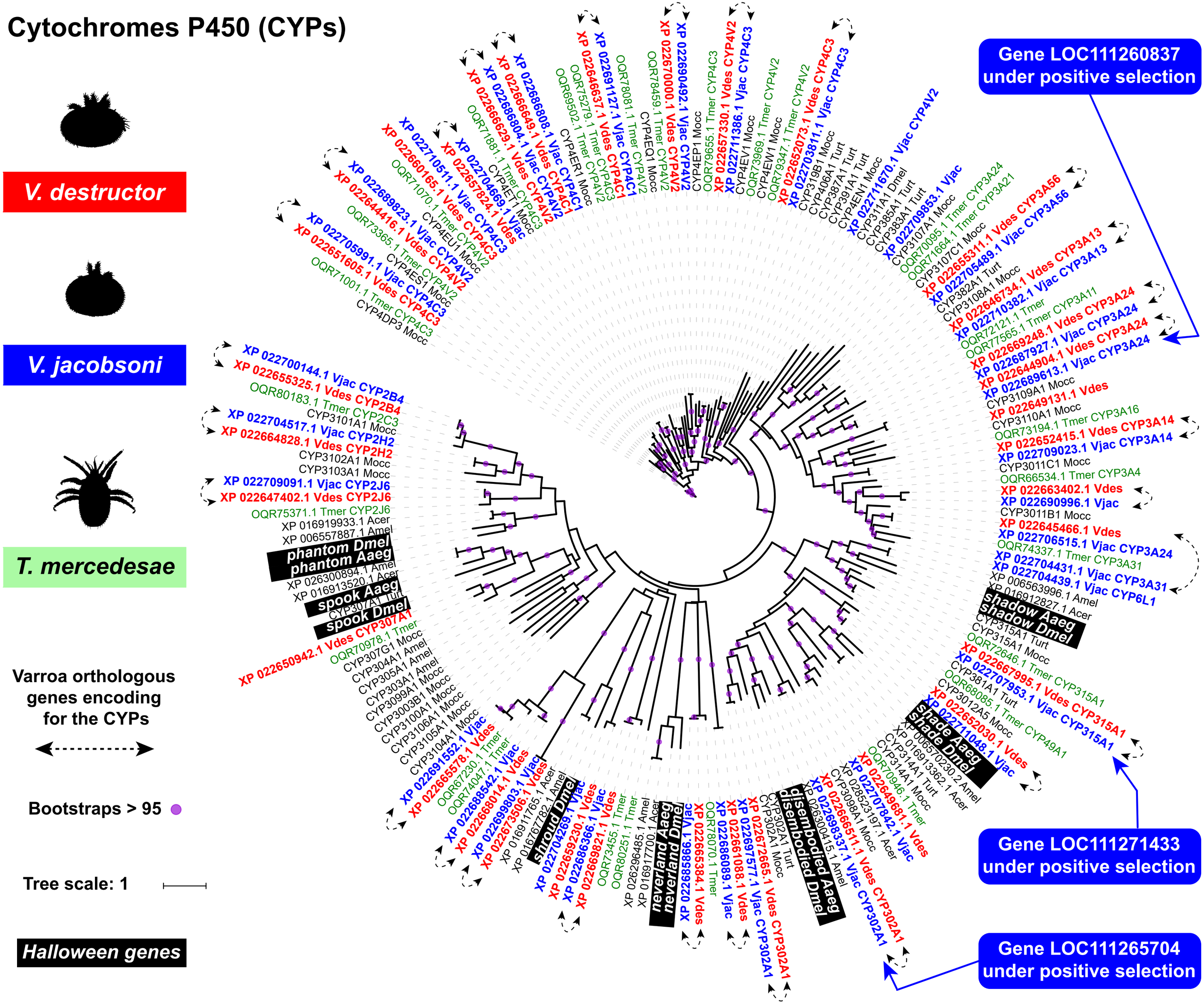
0 Mb
10 Mb
20 Mb
30 Mb
40 Mb
50 Mb
60 Mb
70 Mb
80 Mb
duplicated only in V. destructor
duplicated in both Varroa
chromosomal localization of the orthologs duplicated genes
B
DNA-templated transcription elongation
regulation of cell killing
locomotory behavior
membrane permeability
response to external stimulus
cell-cell adhesion
behavior
cell killing
glycerolipid metabolic process
organophosphate catabolic process
rythmic process
nitrogen compound process
cation transport
lymphocyte mediated immunity
GO terms count
2
4
6
Semantic space y
Semantic space x
5
0
-5
V. destructor
V. jacobsoni
duplicated in both Varroa


Gene LOC111245193 under positive selection
Gene LOC111270396 under positive selection
V. destructor
V. jacobsoni
T. mercedesae
Varroa orthologous genes encoding for the IGR proteins
Bootstraps > 95
Tree scale: 1
Ionotropic glutamate receptors (IGRs)

Cytochromes P450 (CYPs)