Varroa mites escape the evolutionary trap of haplodiploidy

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Abstract

Genetic diversity is essential for populations adapting to environmental changes. Due to genetic bottlenecks invasive species have reduced genetic diversity. However, they must quickly adapt to changes in new environments, potentially including anthropogenic countermeasures. This paradox raises a fundamental question: how do species adapt to changes while having low genetic diversity? The invasion paradox is more pronounced for some species. Parasites go through additional bottlenecks between hosts. Haplodiploid species have a lower effective population size as males can inherit and transmit only half of their mother's genome. Trying to solve this paradox, we investigated inheritance in the Varroa mite (*Varroa destructor*), a well-studied invasive parasite of honey bees fitting all of the above criteria. By following the flow of alleles across three-generation pedigrees we found that Varroa, so far believed to be haplodiploid, is actually not. Rather, it has a unique reproductive system in which females clonally produce functionally diploid sons. While males lose somatic DNA during development, they can transmit either copy of the maternal genome to their daughters. This enhances female fitness, particularly under sib-mating typical of Varroa. We suggest this allows a greater effective population size relative to haplodiploidy and, thus, an increased evolutionary potential. This reversion to diploidy is a singular example of escaping the 'evolutionary trap' of haplodiploidy, which is believed to be an evolutionary stable end state. Plasticity in reproductive systems could be more common than assumed, and may potentially explain the remarkable resilience and high adaptivity of Varroa and other invasive parasites.

Significance

Varroa mites have driven the collapse of honey bee populations since their worldwide spread in the middle of the 20th century. Despite repeated genetic bottlenecks, Varroa has adapted to diverse environments and has overcome many pesticides. Using pedigree analysis, we found that Varroa re-evolved diplodiploid reproduction from an evolutionary history of haplodiploidy. Diplodiploidy permits a higher effective population size and evolutionary potential, likely facilitating Varroa's ongoing success. Females produce males clonally, passing on their entire genomes. Varroa is a singular exception to the theoretically and empirically observed rule that, one evolved, haplodiploidy is an evolutionarily stable

end state (an 'evolutionary trap'). Novel mechanistic studies of even well-known organisms can lead to surprising insights into the evolutionary plasticity of reproductive systems.

Introduction

Genetic diversity is the basis for biodiversity, being required for species to evolve in response to environmental changes (1). For a given selection coefficient, the effectiveness of natural selection *vs.* genetic drift is determined by the effective population size (2). Selection dominates at higher population sizes, yet in small populations, beneficial alleles can be lost due to drift. Biological invasions are often accompanied by genetic bottlenecks that reduce effective population size. Parasites are particularly at risk, as they often experience bottlenecks as part of their life cycle: an introduction to a new host often involves one or a few individuals, and the host-parasite population dynamics also involve frequent and drastic changes in the population's size (3). In addition, because of difficulties involved in finding unrelated mates within a host, many parasites frequently inbreed, which further decreases genetic variability (4–6). Although all the above scenarios are expected to decrease the effective population size, by definition, invasive parasites are successful species that are highly adaptable to different environments, hosts, and in many cases, the chemical pesticides used to control them (7, 8). This 'genetic paradox of invasion' (9, 10) raises the question of how invasive species with expected low genetic diversity are so successful.

A few solutions have been suggested for this paradox. These include physiological explanations (11–13), and multiple invasions that compensate for low genetic diversity (13–15). Occasionally, organisms evolve unusual reproductive strategies that help them avoid inbreeding. This is the case of the ant *Vollenhovia emeryi*, in which males and females constitute genetically distinct clonal lineages that combine to produce sterile workers but otherwise do not intermix (16). Detecting such mechanisms requires controlled crosses, which are often difficult if not impossible, especially in non-domesticated organisms, suggesting that many unusual reproductive modes remain to be discovered.

Here we examined the reproductive biology of the Varroa mite, *Varroa destructor*, a global invasive parasite of honey bees. The mites experienced a range of genetic bottlenecks. They

went through a genetic bottleneck when switching hosts from their original host, the eastern honey bee (*Apis cerana*) to the western honey bee (*Apis mellifera*) (17). They also lose genetic diversity when colonizing new hives. Lastly, they routinely sib-mate in small families (typically one male and three to four females) (18, 19). This mating behavior further increases the strength of genetic drift and makes natural selection less effective. In addition, Varroa is believed to be haplodiploid, which further reduces the effective population size (20, 21). Despite the above, the mite shows high adaptability: it efficiently switches between host species (22–24), and can rapidly evolve resistance to pesticides via various mechanisms (25). While a high level of adaptability explains the mite's successful global spread, the mechanisms underlying this adaptability remain puzzling in the light of regular genetic bottlenecks, routine inbreeding, and male haploidy.

Varroa is believed to be haplodiploid, whereby sex is determined by arrhenotokous parthenogenesis, that is, fertilized eggs develop into diploid females, while unfertilized eggs develop into haploid males (19, 26–28). This type of reproductive system is believed to be stable, sometimes being referred to as an 'evolutionary trap', suggesting limited potential for an evolutionary reversion to diploidy (29, 30). Varroa's ploidy was inferred from two karyotyping studies concluding that it is haplodiploid, with seven pairs of chromosomes (31, 32). However, as those studies examined Varroa embryos, sex could not be directly determined. Those studies also differed in key findings, such as chromosome shape: De Ruijter & Pappas (32) found that all chromosomes are metacentric, while Steiner et al (31) found that they have three submetacentric and four acrocentric chromosomes. Häußermann et al. (19) found that unmated females can produce males, consistent with arrhenotokous parthenogenesis *vs.* paternal genome elimination, which is found in many other mites. Cruickshank and Thomas (33) used phylogenetic reconstruction to infer that Varroa evolved from diploid ancestors, via intermediate pseudo-arrhenotokous species (note: while Varroa was included in this study, its haplodiploidy was assumed based on the fact that all members of its family are haplodiploid). Theory suggests that reversal to diploidy from arrhenotoky is unlikely (30), and, indeed, no reversals of arrhenotokous sex determination are known to date (34). Yet, despite strong circumstantial evidence suggesting the existence of arrhenotokous parthenogenesis in Varroa, no study has

112 examined Varroa's mode of genetic inheritance, making its success in spite of genetic
113 handicaps somewhat of a mystery. Here, we explicitly examine this species' mode of genetic
114 inheritance using multi-generational pedigrees.
115
116 **Results**
117 Three lines of evidence suggest that Varroa mite males are functionally diploid: [1] they
118 inherit two maternal alleles evident in their genotypes (Fig 1A) [2], they randomly transmit
119 these genotypes to their daughters (Fig 1B), and [3] these genotypes express at equal levels
120 (Fig 2). Interestingly, flow cytometric investigation suggests that, while on average, females
121 have roughly twice as much genetic material as males, the male somatic ploidy is more
122 variable and ranges from haploid to diploid (Fig S1).
123
124 *Most males are produced parthenogenetically, preserving almost all of their mothers' diploid*
125 *genotypes.*
126 The majority of F2 males inherited almost all of their mother's genotype (Fig 1A). This did
127 not appear to be the case for all males, some of whom had significant genetic contributions
128 from their fathers. To facilitate the analysis, we separated the males into two groups:
129 parthenogenetic and other. Below we focus our analysis on the parthenogenetic males,
130 which made up 20 out of the 26 F2 males in our sample, which had a high maternal
131 genotype proportion out of the total number of variants (median = 0.98) (Fig 1A). There
132 were too few of the other males to infer any generalizations, other than to note the
133 possibility that some males were sexually produced from a fertilized egg. By contrast, F2
134 female mites randomly inherited both parents' genotypes (Fig 1A).
135
136 *Males transmit their two alleles to their daughters with equal probability*
137 Consistently, males' maternal inheritance was also observed for the heterozygous variants.
138 Looking at the cross between a heterozygous F1 female (AB) and a homozygous F1 male
139 (AA), we found that 97% of the variants in F2 males maintained their mother's
140 heterozygosity (Fig 1B). Furthermore, males not only inherited two alleles, but they also
141 randomly transmitted each allele to their daughters. Looking at the cross between a
142 homozygous female (AA) and a heterozygous F1 male (AB), we observed that 50% of the

143 variants in F2 females were homozygous (like mother) and another 50% were
144 heterozygous (like father). F2 male offspring of the same cross were homozygous (AA), just
145 like their mother (Fig 1C).
146
147 *Males carry and express two alleles*
148 Varroa males not only inherited and transmitted two alleles, but could also express both
149 alleles, as evident from publicly available RNAseq data (Fig 2, Table S1) showing that
150 Varroa mite males and females had similar levels of heterozygosity in DNA (A, females:
151 0.20±0.12, males: 0.20±0.14) and RNA data (B, females: 0.86±0.03, males: 0.90±0.08). In
152 contrast, in honey bees, a known haplodiploid species, females had higher levels of
153 heterozygosity in DNA (C, females: 0.48±0.02, males: 0.12±0.09) and RNA (D, females:
154 0.47±0.02, males: 0.10±0.03).
155
156 *Males' somatic ploidy is variable*
157 Male heterozygosity raised the possibility that males are actually diploid instead of haploid,
158 as believed so far. Therefore, we assessed the ploidy of males and females using flow
159 cytometry experiments, in addition to bioinformatics k-mer distribution analysis of the
160 whole genome from a global mite collection (BioProject PRJDB6279, (17)). Flow cytometry
161 experiments showed that on average, females had roughly twice as much genetic material
162 as males in somatic tissues (2.25 and 1.63 for females and males respectively, Fig S1).
163 However, males' somatic ploidy was more variable and ranged from 0.35 to 2.75, while
164 most females' ploidy was higher than 2, and ranged from 2.1 to 2.85 (Fig S1). The higher
165 ploidy of females was observed in all developmental stages, and germline tissues in both
166 sexes were haploid (Fig S2 A and B). K-mer distribution analysis suggested that the level of
167 heterozygosity is similar in males and females (Fig S3).

Discussion

In this study, we found that *V. destructor* has evolved from ancestral arrhenotoky (33) to a novel reproductive strategy with functionally diploid males, albeit being uniparentally produced by females. This strategy has both short-term and long-term potential benefits for Varroa, each overcoming a different evolutionary 'paradox'. In the short term, the production of clonal males increases the females' fitness by assuring transmission of both of her genetic variants. This strategy can overcome the "paradox of sex" by blending the benefits of sexual and asexual reproduction. Namely, females can produce sons containing both copies of their genes, but female eggs are produced meiotically. In the long term, functional diplodiploidy can potentially increase the effective population size (*vs.* haplodiploidy). A higher effective population size imparts greater evolutionary potential, and indeed Varroa has been remarkably adaptable despite repeated bottlenecks exemplifying the "genetic paradox of invasion".

Another evolutionary surprise from this study is the effective loss of arrhenotoky. Once evolved, asymmetric inheritance appears to be in an evolutionarily stable state, sometimes referred to as an 'evolutionary trap' (30). Obstacles to evolving Mendelian inheritance are diverse. One obstacle is mechanistic, in that males would need to re-evolve meiotic sperm production (35). The other obstacle is evolutionary. In order to invade a population, biparental diploid males must carry mutations that allow them to produce diploid sons. However, arrhenotokous females control fertilization and sex ratios, making such an invasion theoretically unlikely (36). How Varroa re-evolved male sperm production is unknown. Recent work from asexual stick insects indicates that male meiotic regulatory mechanisms are preserved in the females (37), so re-evolving male meiosis may not be as difficult as previously thought. The evolution of diploid males in arrhenotokous systems was believed unlikely because females control the sex ratio, and diploid male phenotypes would be unlikely to invade (30). However, Varroa seems to have overcome this constraint by having females solely control the sex ratio, as well as diploid male production. These two points illustrate that the unforeseen complexity of biological mechanisms may challenge long-standing elegant mathematical models.

199 *Peculiarities and outstanding questions in Varroa reproduction.*
200 While our results conclusively illustrate the functional diploidy capabilities of Varroa mites,
201 there remain a number of mechanistic questions about how reproduction in that species
202 actually occurs. Answers to these questions are not critical for the main conclusions of this
203 study – *i.e.*, effective diploidy in Varroa – but provide interesting avenues for future
204 investigation.
205
206 First, the mechanism by which clonal males are produced is unclear. However, it is most
207 consistent with central fusion, a type of automixis involving the fusion of the two products
208 of meiosis I, which tends to largely preserve maternal heterozygosity. The extent of
209 heterozygosity preservation under central fusion is dependent on how much
210 recombination between homologous chromosomes takes place (38). Thus, the small
211 number of males that strongly differed from their mother's genotype (Fig 1A) could have
212 been produced by variation in meiotic recombination rates. However, there were too few
213 such males for us to investigate them in greater detail and arrive at anything but
214 speculative conclusions.
215
216 Second, in contrast to the germ line, which must be diploid, the somatic karyotype of
217 Varroa males is highly variable. How stability in the germline is maintained and the reasons
218 for somatic instability remain unclear. Flow cytometry indicates that, in contrast to
219 females, which are diploid, males exhibit a range of somatic DNA content from haploid to
220 diploid (Fig S1 and S2). This suggests that males start their lives as diploids, which is a
221 necessity for passing on two allelic copies, but they may lose part of their genome
222 subsequently. The mechanisms for genome elimination are unclear, though they may have
223 to do with maintaining the dosage of male-specific genes, which have evolved in a haploid
224 context. Given that both maternal alleles are equally likely to be expressed (Fig 1A and 1B),
225 this elimination process appears to be random, and the male somatic genotype is likely
226 chimeric. Unusually, spermatogenesis in Varroa is an ongoing process during the adult
227 stage, only completing after sperm transfer to females (39, 40). Our findings suggest that
228 genome elimination happens only in the male's somatic tissues, while germlines remain in
229 a similar ratio of the two maternal alleles.

230 Lastly, a major mystery surrounds the mechanisms of Varroa sex determination. Since
231 males and females are both diploid, it is unlikely that genetic mechanisms, such as
232 complementary sex determination, would be at play. Rather, there is likely an epigenetic
233 mechanism at play instead. In Varroa, there is a correlation between offspring sex and
234 fertilization, with fertilized and unfertilized gametes becoming females and males,
235 respectively, so there is the potential for genetic imprinting to play a role.
236
237 *Conclusions*
238 Our work underscores the importance of comprehensive experimental inquiries into
239 diverse reproductive mechanisms, even in organisms that appeared to be well studied.
240 Recent investigations have unveiled a broader spectrum of reproductive strategies than
241 were previously recognized (35), exemplifying the dynamic nature of biological systems.
242 The discovery of functional diploidy in Varroa mites has profound implications for bee
243 health management, potentially explaining this species' adaptability and success as an
244 invader despite its repeated bottlenecks. Our work exemplifies the need for re-evaluating
245 Varroa population dynamics models, particularly the spread and persistence of pesticide
246 resistance mutations, which are the primary impediment to effective Varroa control.
247 Furthermore, Varroa has emerged as a compelling model for the study of the evolutionary
248 dynamics of reproductive systems, offering insights into mechanisms for overcoming
249 evolutionary constraints and the intricacies of sex determination. These findings open
250 promising avenues for future research, contributing to a deeper understanding of
251 evolutionary innovation and reproductive plasticity in Varroa and beyond.
252
253 **Materials and methods**
254 *Honey bees and mites*
255 For the pedigree study: Mites and honey bees (*Apis mellifera* L.) were obtained from
256 colonies at the Onna village apiary and the experimental apiary of Okinawa Institute of
257 Science and Technology (OIST) in Okinawa, Japan (26.477 N 127.835 E). All colonies used
258 in the experiment were maintained without any treatment against Varroa and received
259 sugar solution and 70% pollen cakes as supplemental feed when necessary. Experiments
260 were conducted between July and November 2020.

261 For the flow cytometry study: Mites were freshly collected from drones and worker brood
262 frames of *A. mellifera* colonies at the Texas A&M University Honey Bee Lab apiary (Janice
263 and John G. Thomas Honey Bee Facility). Mites were collected in June, July, and December
264 2022. Capped cells containing red-eye pupae (day 15) to emerging adults (day 19-20) were
265 carefully opened and inspected for single infestation, following standard methods and
266 procedures (41). The developmental stages and identification of vigorous specimens were
267 conducted using a soft and fine paintbrush within a petri dish under a Leica macro-
268 binocular.
269
270 *Artificial infestation for Varroa pedigree construction*
271 To obtain a three-generational mite pedigree (F0, F1, and F2), we used a semi-natural
272 infestation system. This started with the collection of a naturally infesting mated Varroa
273 female (F0). Her adult daughter (F1) was then artificially introduced into a honey bee pre-
274 pupal cell and allowed to commence the reproductive phase of its life cycle. The F1 mite
275 and her offspring (F2) were then collected, at the end of the reproductive cycle. For a
276 detailed description of the experimental design, see Fig 3 and the sections below. Using this
277 approach, we could genotype all members of the pedigree, except for the male that mated
278 with the F0 female.

279 Collection of naturally infesting families (F0 and F1 generations)

280 To obtain mites from the first two generations (F0 and F1), naturally infested families were
281 collected from within the cells of 18- to 20-day-old worker bee pupae. At this
282 developmental stage of the honey bee host, there is typically one dark brown female mite
283 (the foundress, F0), one adult male (the foundress's son, F1), at least one light-colored
284 female (the first foundress's daughter, F1), a few nymphs females, and an egg (26). We
285 determined each mite's sex and developmental stage according to external morphology and
286 cuticle coloring based on (42) for the color index, please see Fig S4. To ensure that all
287 offspring belonged to a single family, we thoroughly examined the cell content to confirm
288 that it was infested by a single foundress mite and that no other adults were present.
289 Except for the adult female daughters (F1), the rest of the family members were preserved
290 in a separate microcentrifuge tube kept at -20 °C for later DNA extraction. The adult F1

291 daughters were easily distinguished from the F0 foundress mite by their lighter cuticle
292 color (color index 0-2, see Fig S4), and were assumed to have already copulated with their
293 brother, the F1 male, by the time of the collection (19). Next, the F1 young adult females
294 were prepared to be artificially introduced into a freshly capped 5th instar larva cell to start
295 their first reproductive cycle.

296 Incubation ("phoretic phase") and artificial infestation of F1 female mites

297 Based on our and others' observations (43), the survival and fecundity of young adult mites
298 increase if they stay for a few days on an adult nurse bee before starting their first
299 reproductive cycle. This incubation period is presumably imitating the "dispersal stage", in
300 which the adult female mite infests an adult honey bee (43, 44). Therefore, the collected
301 young adult females (F1 generation) spent three days on a nurse bee before infestation.
302 However, during the experiment, we noticed that the very young adult daughter's cuticle
303 was light and fragile, and most of the mites did not survive when they were transferred
304 directly from the pupae onto a nurse bee. Therefore, we let the young mites first feed on a
305 white-eyed pupa in a gelatin capsule for one to four days until their cuticle was sclerotized
306 and darker (color index 3-4, see Fig S4). After this intermediate period, the mites were
307 marked on their dorsal idiosoma (PC-1MR POSCA, Japan), and transferred onto an adult
308 nurse bee in a cage for another three days, for the above-mentioned "dispersal stage". In all
309 incubation periods, the mites were kept in a controlled dark environment, imitating hive
310 conditions (34-34.5 °C, 60-75% RH).
311
312 After the artificial "dispersal phase", the mites were brushed off the bees using a fine paint-
313 brush following anesthesia by a 20 seconds exposure to CO_2, 0.2mPa at 10 L/hour, and
314 placed on a moist filter paper to avoid desiccating till infestation. The mites were then
315 introduced to a worker bee cell, one mite per cell, as described by (41). In detail, six hours
316 before infestation, cells containing a 5th instar larva (about to be sealed) were marked on
317 an acetate sheet attached to a frame from a mite-free hive. The frame was then placed back
318 into the hive so that the bees could seal the cells. Five hours later the frame was taken into
319 a warm room (25-28 °C) for the F1 females to be introduced into the freshly sealed cells.
320 For the artificial infestation, a precise incision (3-5 mm) was made at the margin of the cell-

321 capping using a surgical blade (N 11). After the incision was made, the cell-capping was
322 slightly lifted, just enough for the mite to crawl under the capping and into the cell. The
323 mite was then placed at the entrance of the cell using a moist fine paintbrush, with its head
324 facing the cell opening. After the mite had entered the cell, it moved under the larva to the
325 cell bottom. The cap was then resealed by gently pushing it down using the back end of the
326 paintbrush. The infested frame was then incubated in a dark controlled environment (34-
327 34.5 °C, 60-75% RH), and emerging bees were removed daily.

328 Collection of an artificially infested family (F2 generation)

329 Ten days after the F1 mites infestation, the cells were opened, and their contents were
330 examined. The family members' sex, developmental stage (adult, nymph, or egg), and
331 viability were recorded. To assure the parenthood of the offspring (F2), double-infested
332 cells and unmarked mites were not included in the final analysis. In total, out of 78
333 successful infestations, 14 were double-infested by a non-marked mite. Each individual was
334 snap-frozen in a separate tube at -20 °C for later DNA extraction. In total, we collected 223
335 mites belonging to 30 families. Each family was composed of a foundress adult female (F0),
336 her male and female adult offspring (F1), and their F2 offspring (at least one male/female
337 mite in various developmental stages).
338
339 *Genomic analysis of the Varroa pedigree*

340 DNA extraction, library preparation, and sequencing

341 Genomic DNA was extracted from each single mite following the protocol described
342 previously (22). In brief, after surface sterilization using absolute ethanol, each mite was
343 crushed in a 1.5 mL microcentrifuge tube dipped in liquid nitrogen. The crushed mite was
344 then processed using QIAamp DNA Micro Kit (© Qiagen) following the manufacturer's
345 instructions and eluted in 20 µL of water. Total dsDNA quantity was measured using a
346 Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and DNA quality was
347 verified using a Bioanalyzer 2100 with high-sensitivity DNA kits (Agilent, Japan). Short-
348 inserts of 150-bp paired-end libraries were prepared for each individual using a Nextera
349 XT DNA Library Preparation Kit (Illumina, Japan) and 16 PCR cycles. cDNA was then

350 cleaned and size-selected using CA-magnetic beads (Dynabeads® MyOne Carboxylic Acid,
351 Invitrogen), and 11-11.5% PEG 6000 (Sigma-Aldrich, Japan). The library quality was
352 measured using a Qubit™ 4 Fluorometer with an Invitrogen dsDNA HS Assay Kit, and its
353 size was assessed using a 4200 Tapestation with a high-sensitivity D5000 kit (Agilent,
354 Japan). Libraries were run on a NovaSeq6000 in 150 bp x 2 paired-end mode (Illumina,
355 Japan) at the OIST Sequencing Center. All biosamples are available in the Sequence Read
356 Archive (SRA) under the accession number PRJNA794941. Biosample details including sex,
357 developmental stage, pedigree information, and sequencing coverage, are provided in
358 Table S2.

359 Reads mapping, variant calling, and filtering

360 We mapped all reads to the *V. destructor* reference genome [GCF_002443255.1] (17) using
361 the very sensitive mode of NextGenMap v0.5.0 (45). Reads were sorted and duplicates were
362 removed using SAMtools (46). We called variants against the reference genome using
363 FreeBayes v1.1.0 (47) with the following parameters: minimum alternative allele counting
364 = 5, minimum alternative allele fraction = 0.2, minimum mapping quality = 8, minimum
365 base quality = 5, and using the four best SNP alleles. The variant calling resulted in a total of
366 73,903,468 variated sites. As our population consisted of closely related individuals (mite
367 families from the same apiary, in a high-inbreeding system), we did not expect to detect
368 many variants. Therefore, we set the minor allele frequency threshold to 0.2, which
369 includes sites with a minor allele frequency of 20% or more, to exclude any rare variants
370 due to mutations or possible mapping errors. We included variants only for the seven
371 chromosomes (excluding the mitochondrial DNA and unscaffolded regions). In addition, we
372 filtered for the following site quality parameters: we included only bi-allelic sites, a depth
373 range between 16-40, and maximum missing data per site = 0.5. The variants' quality
374 distribution showed a clear bimodal distribution with a cutoff at ~10,000 (Fig S5). To
375 ensure the quality of the variants for the genetic inheritance analysis, we chose a strict
376 filter of minimum quality of 15,000. Filters were applied using VCFtools v0.1.12b (48)
377 resulting in 33,925 final variants in 223 individuals. The pipeline from fastq files down to

378 the VCF file was constructed using Snakemake (49), and codes are available and
379 reproducible on GitHub [https://github.com/nurit-eliash/Varroa-pedigree-study].
380
381 *Genotype validation using Sanger sequencing*
382 Several genotype calls obtained from whole genome sequencing were validated by Sanger
383 sequencing using the same genomic DNA extracted. For the validation, we randomly
384 selected three genes, located on different chromosomes, each containing at least one
385 variant site. In total, we re-sequenced 14 individual mites from four families on six variant
386 sites. The sequenced region was located on the exons of three genes: alpha-mannosidase
387 2x-like (LOC111243621 on chromosome NW_019211455.1), 5-oxoprolinase-like
388 (LOC111252938, on chromosome NW_019211459.1), and probable E3 ubiquitin-protein
389 ligase RNF144A-A (LOC111254119, on chromosome NW_019211460.1). Primers were
390 designed with the NCBI primer design tool (utilizing Primer3 and BLAST), with default
391 parameters and product size set to 300-1,000 bp. Primer sequence, product length, and
392 gene accession numbers are provided in Table S3. The size of the PCR product was checked
393 by running in 1% agarose gel (135V, 20 min), purified, and size selected using the solid
394 phase reverse immobilization (SPRI) method. The purified PCR products were Sanger
395 sequenced on a ThermoFisher SeqStudio Genetic Analyzer using the original reverse
396 primer and BigDye® Direct Cycle Sequencing kit (Thermo Fisher Scientific), following the
397 manufacturer's instructions. We then aligned the reverse complementary sequences to the
398 Varroa reference genome (GCF_002443255.1), to detect and validate the variant sites using
399 Geneious Prime 2022.1.1. (https://www.geneious.com). Sequence alignments and the
400 validated sites can be seen in Fig S6.
401
402 *Ploidy assessment*

403 Flow cytometry

404 We used flow cytometry of the Varroa mite nuclei to assess the male and female mites'
405 ploidy during development from nymphs to adults. Live mites were freshly collected and
406 snap-frozen in liquid nitrogen before being processed. Samples were ground using 15
407 strokes of the "A" pestle in a 2 mL Kontes Dounce tissue grinder and filtered through a

408 40μm nylon mesh. Each sample consisted of an individual mite: for immature mites (larvae
409 and nymphs), a whole larval body, and for adult mites, dissected somatic or germinal
410 tissues. The DNA in the nuclei released by grinding was stained for 2 hr under dark
411 refrigeration with 25 μg/mL propidium iodide. The mean red PI fluorescence of the stained
412 nuclei was quantified using a Beckman-Coulter (Brea, CA) CytoFlex flow cytometer with a
413 solid-state laser emitting at 488 nm. As a standard, we used nuclei from the brain of
414 *Drosophila virilis,* 1C =328 Mbp (50). The total quantity of DNA in the sample was
415 calculated as the ratio of red fluorescence of the sample/standard (mean red fluorescence
416 of the 2C peak of the sample divided by the mean fluorescence of the 2C peak of the
417 standard), times the 1C amount of DNA in the standard. In total, 75 samples were evaluated
418 of different sex and developmental stages. We scored these samples to produce biological
419 replicates, to calculate the standard error of the mean genome size estimate. To identify the
420 ploidy level of a Cytometric DNA peak (51), genome size is reported as 1C, the mean
421 amount of DNA in Mb in one copy of a single complete genome.

422 K-mer distribution

423 We used the Smudgeplot method (52) to visualize and estimate the ploidy of male and
424 female genomes by analyzing heterozygous k-mer pair, from the raw reads of a male
425 Varroa mite (BioProject PRJDB6279, runs DRR121854 and DRR124352 for male and
426 females respectively).
427
428 *Heterozygosity levels in DNA and RNA of Varroa and of a confirmed haplodiploid species (Apis*
429 *mellifera)*
430 To test for heterozygosity levels in the DNA and RNA of Varroa mites and honey bees, we
431 ran the same pipeline that was used to generate Varroa-pedigree results, using the known
432 haplodiploid species genome of *Apis mellifera*, as input files. In addition, to test if Varroa
433 was not only carrying but also expressing both sets of alleles, we checked heterozygosity
434 levels in Varroa RNA-seq reads. The input files were as follows: For Varroa RNA, we used
435 RNA-seq reads from Bioproject PRJNA380433, of the Varroa Genome Sequencing
436 Consortium done at the USDA, which includes five female mites (three adults and two
437 nymphs) and two males (a nymph and an adult). For honey bee DNA, we used genome

438 sequencing reads from accession PRJNA311274 (53), the SeqApiPop project to characterize
439 the genetic diversity of honey bees throughout France, which included six libraries of
440 haploid males and 30 libraries of diploid females from the same colony. For the honey bee
441 RNA, we used RNA-seq reads from bioproject PRJNA689223, which includes eight females
442 and nine males. We adjusted the pipeline to use the *A. mellifera* reference genome
443 (amellifera_HAv3 [GCF_003254395.2]), and 16 chromosome pairs. Other than these
444 adjustments, all pipeline codes were kept as in the original Varroa genome analysis
445 described in the Reads mapping, variant calling, and filtering section.
446
447 *Statistical analysis*
448 All analyses were carried out in the R statistical environment (54). All bioinformatic
449 analyses are available and reproducible directly from the online supplementary data
450 [https://github.com/nurit-eliash/Varroa-pedigree-study].
451
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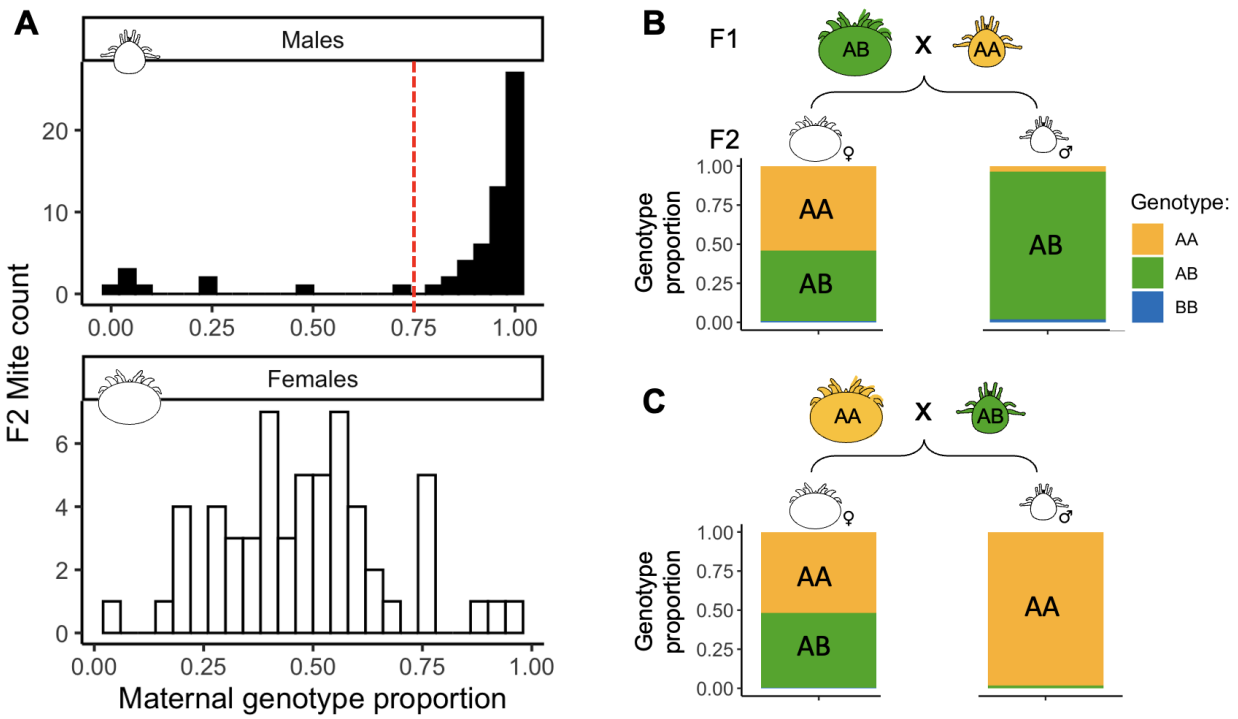


583

584 **Figure 1. F2 mites genotype proportion crossing heterozygous and homozygous F1 mites. A.**
585 **Distribution of maternal genotype proportion in F2 mites shows that male genotypes are almost**
586 **identical to their mothers and that females are produced sexually**. Results are pooled variants of 23
587 males and 19 females from four reciprocal F1 crosses: heterozygous females (AB) x homozygous male (BB or
588 AA), and homozygous females (AA or BB) x heterozygous male (AB). F2 female's distribution is normal (W =
589 0.985, p-value = 0.674, Shapiro-Wilk normality test, mean = 0.48). F2 male's distribution is skewed to the
590 right, with median = 0.98 and non-normal distribution (W = 0.544, p-value = 1.4e-12, Shapiro-Wilk normality
591 test). While most males have their mothers' genotypes, there appears to be some plasticity in the male
592 production mechanism, and a small number of males are different. For further analyses in panels B and C, we
593 discarded males with less than 0.75 identity to their mothers to focus on the most common reproduction
594 mode (red dashed line). **B. Males inherit two alleles from their mothers.** F2 mites genotype proportion
595 crossing F1 heterozygous females (AB) and homozygous males (AA). The results are based on 4,942 pooled
596 variants from 15 F2 females and 5,561 variants from 12 F2 males. **C. Males randomly transmit two alleles**
597 **to their daughters.** F2 mites genotype proportion crossing F1 homozygous females (AA) and heterozygous
598 males (AB). The results are 2,465 pooled variants from 14 F2 females and 1,949 from 14 F2 males. For the
599 rest of the crosses, please see supplementary figures in the link: https://rpubs.com/Nurit_Eliash/1002198.

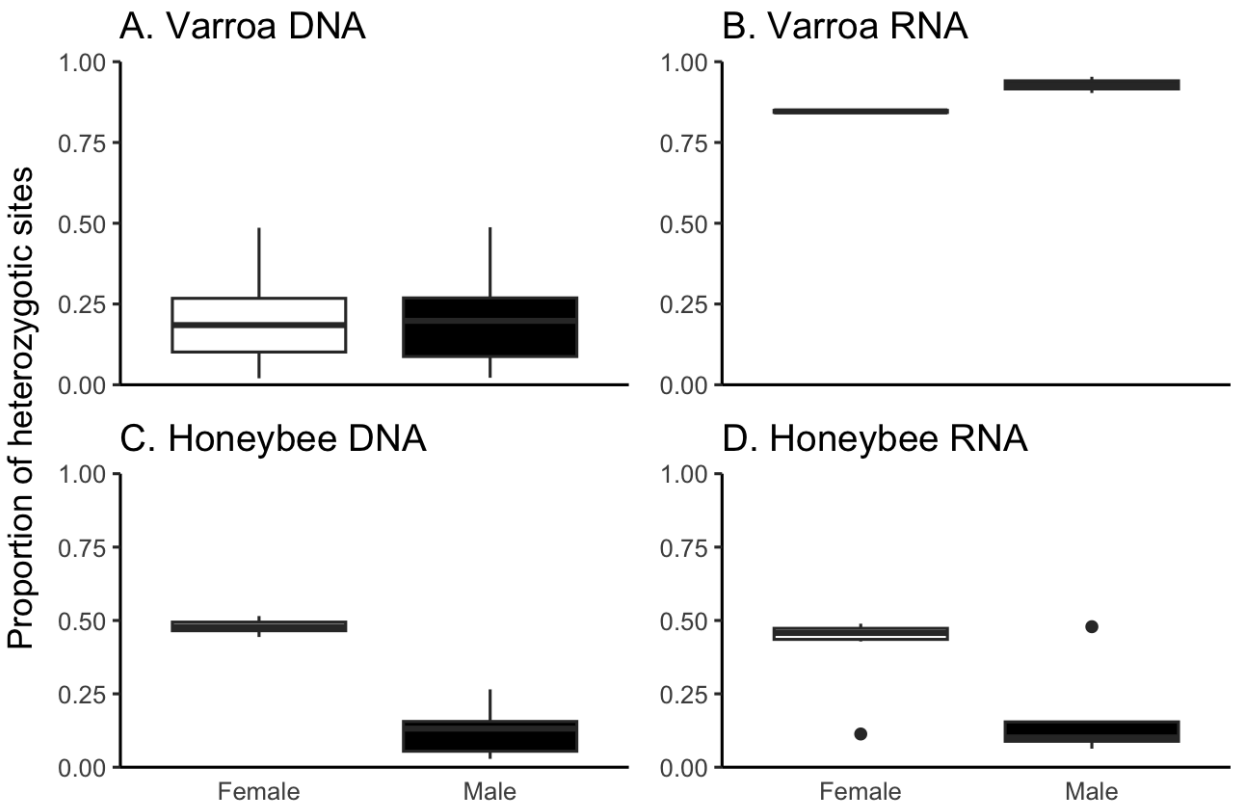


Figure 2. Varroa males carry and express two alleles, like female mites. Varroa mite males and females have similar levels of heterozygosity in DNA (**A**, females: 0.20±0.12, males: 0.20±0.14) and RNA (**B,** females: 0.86±0.03, males: 0.90±0.08). honey bee females have higher levels of heterozygosity than males, in both DNA (**C,** females: 0.48±0.02, males: 0.12±0.09) and RNA (**D,** females: 0.47±0.02, males: 0.10±0.03).

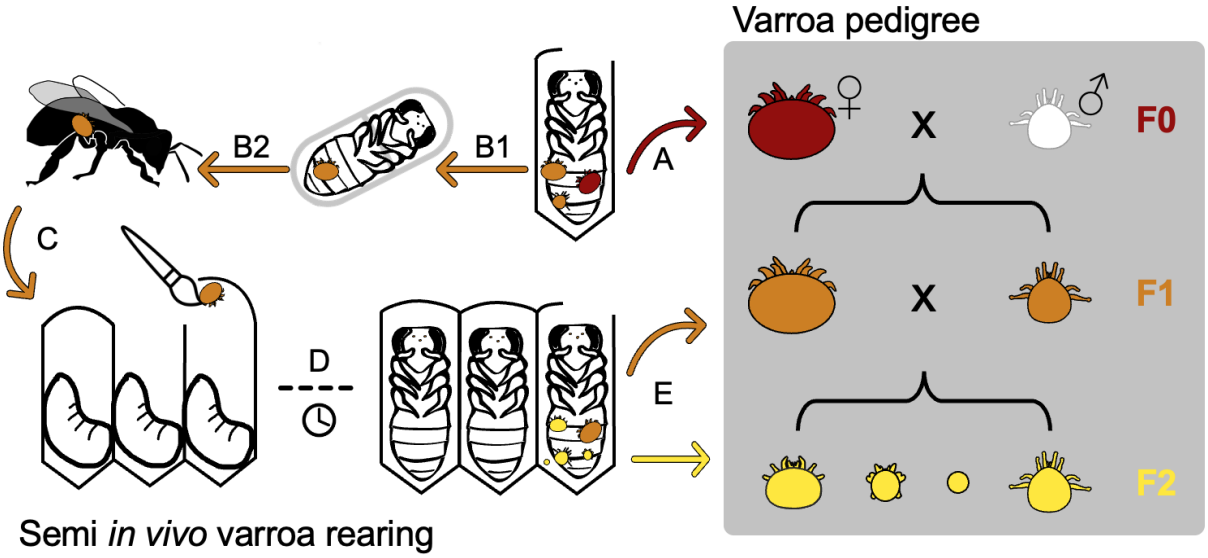
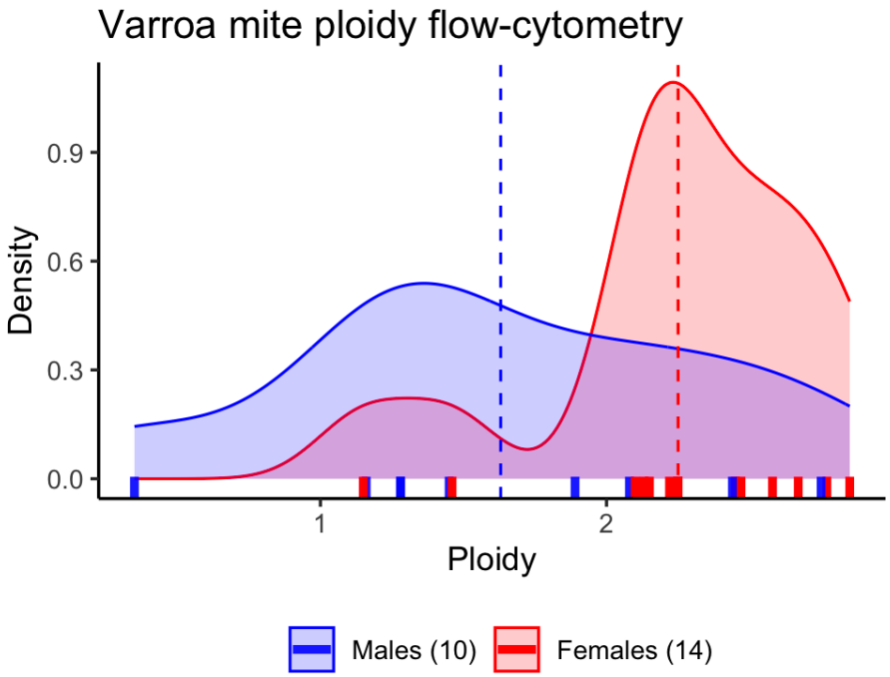


Figure 3. Pedigree experimental design. A three-generation Varroa pedigree (F0-in red, F1-in orange, and F2-in yellow), was constructed by the following steps: **A.** Collecting a naturally infested family: foundress female mite (F0), and her two adult offspring: male and female F1 mites (F0 male's genotype was not possible to study because the F0 female was previously mated, typically by a single male); **B.** "Incubation phase": (B1) Transferring the young F1 female onto a bee pupa for 1-4 days, till cuticle sclerotized and darkened; (B2) Transferring F1 female onto a nurse adult bee for another 3 days; **C.** Introducing the matured F1 female to a freshly capped 5th instar larva cell; **D.** F1 female reproducing inside the bee cell; **E.** 10 days post introduction, collecting the artificially infested family: F1 female and her F2 offspring. This design provided known parents and genotypes for the F1 and F2 generations, allowing a detailed examination of inheritance.

617 **Supplementary information:**



618

619 **Figure S1.** Female mites are mostly diploid, while male mites are more variable. Ploidy assessment
620 of mites' whole body was done using flow cytometry. The ploidy averages are 2.25 and 1.63 for
621 females and males respectively (dashed lines).

622

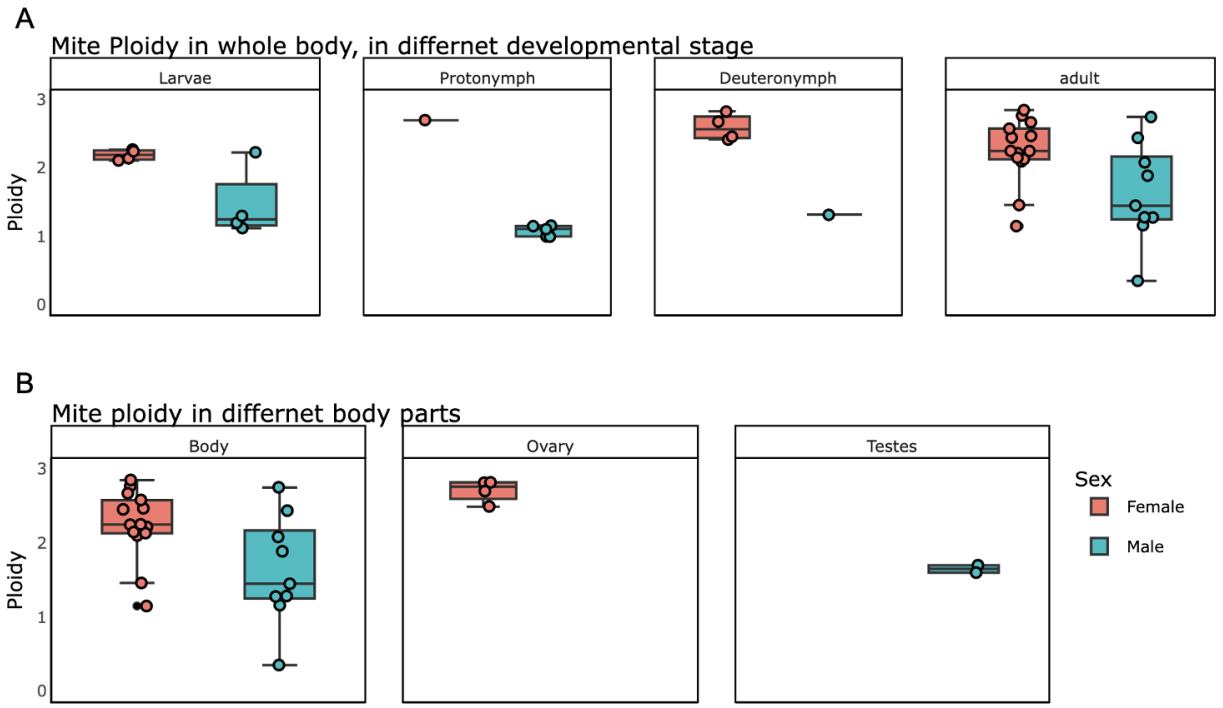


Figure S2. The higher ploidy of females was observed in all developmental stages

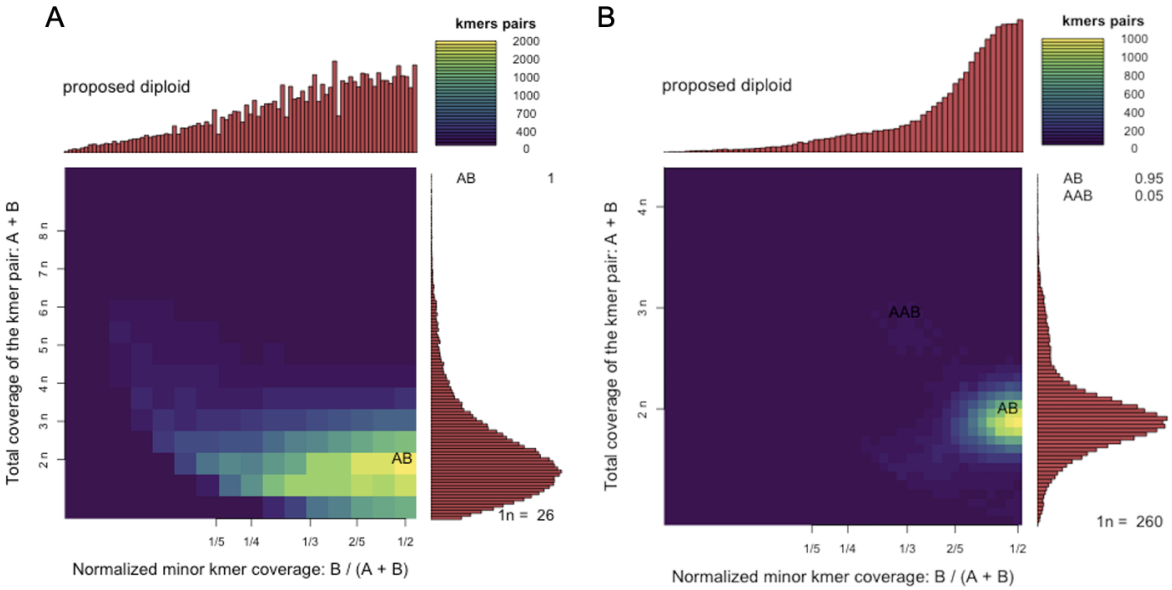


Figure S3. K-mer analysis suggests males and females are diploid or at least carry two sets of alleles. Smudgplot analysis (Ranallo-Benavidez et al. 2020), for female **A**, and male mites, **B**. The male dataset has much higher coverage, but both genomes have a similar signature of heterozygosity.

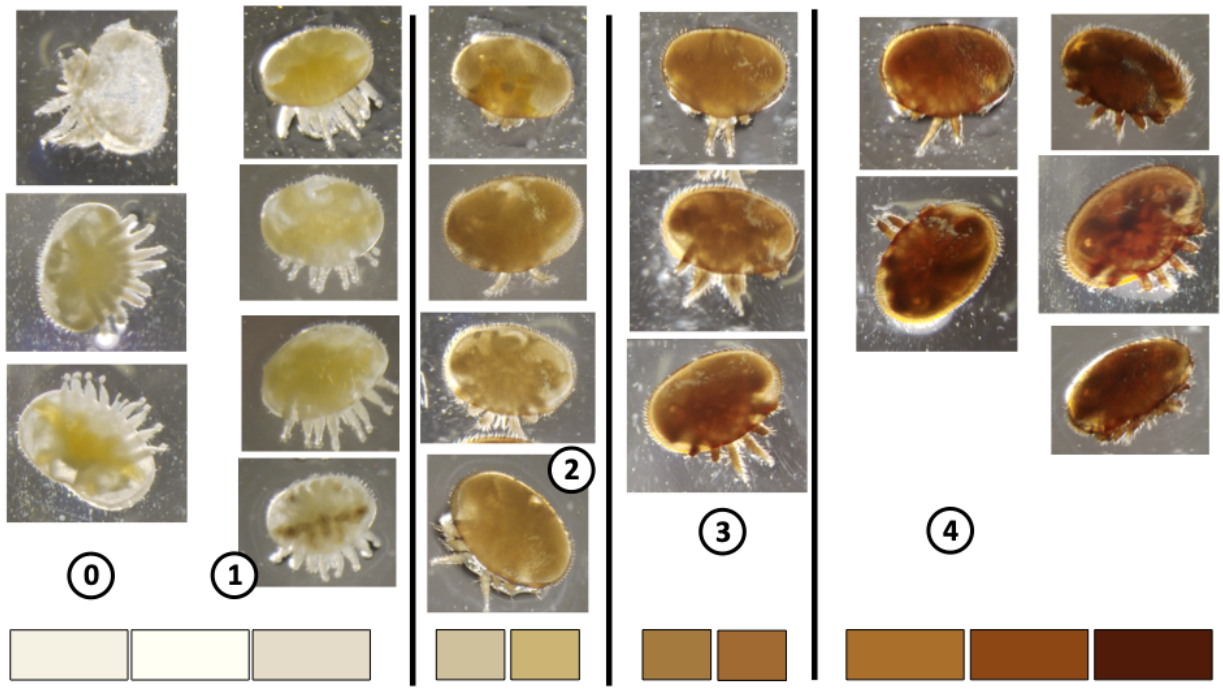


Figure S4. The color scales of Varroa adult female mites, from light white (0) to dark brown (4). Adult female mites in the color of (4) were considered Foundress mites and were used as F0 generation in the artificial infestation experiment. Adult female mites in the colors between 2 and 3 were considered adult daughters of F0 and were used as the F1 generation. Photographs by Maeva Techer.

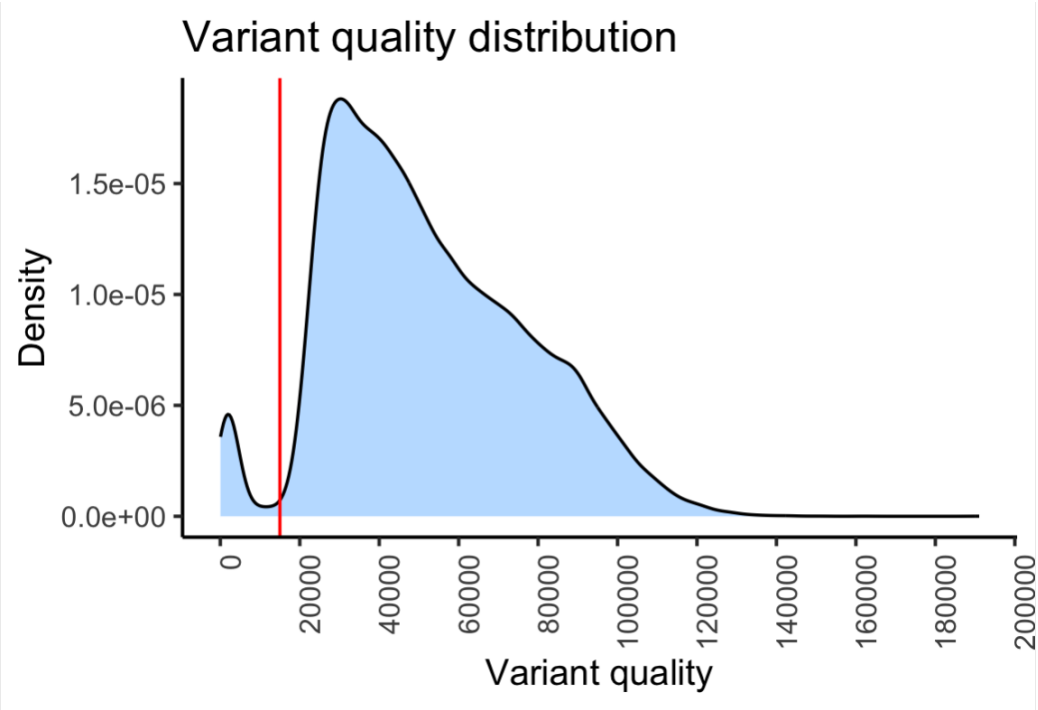


Figure S5. Quality distribution of variants filtered for a minimum quality of 40. The plot shows a clear bimodal distribution with a cutoff at ~10,000. To ensure the quality of the variants for the genetic inheritance analysis, we chose a strict filter of minimum quality of 15,000.

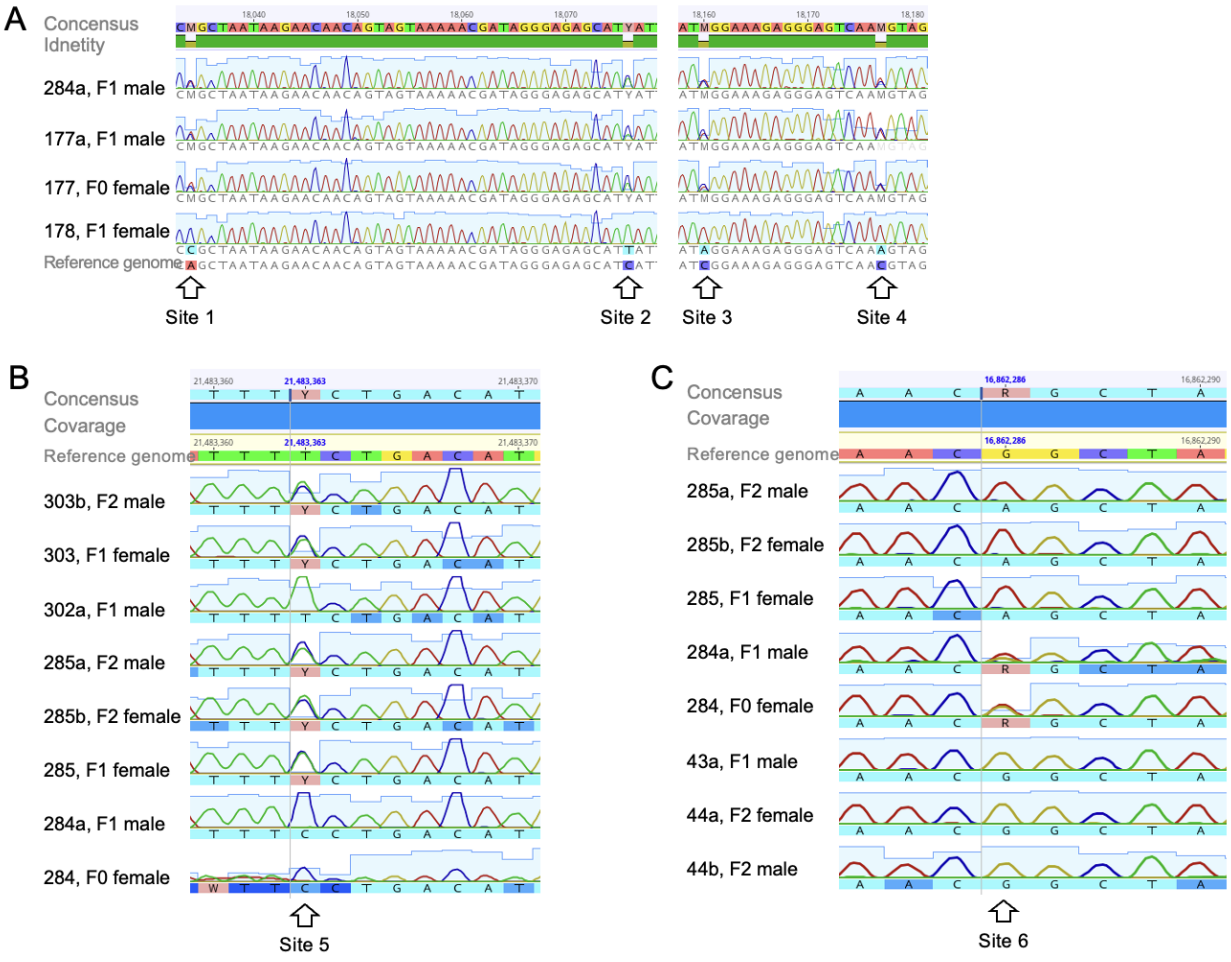


Figure S6. Alignment of Sanger-sequenced regions amplified using mite's DNA from different families as a template. The amplicon sequences were aligned to Varroa mite gene, from the reference genome. (A) Amplicons of alpha-mannosidase 2x-like gene (LOC111243621), amplified on the DNA template of two adult males (284a, 177a) and two adult females (177 and her daughter 178); (B) Amplicons of 5-oxoprolinase-like gene (LOC111252938), amplified on the DNA template of family 302, and family 284; (C) Amplicons of probable E3 ubiquitin-protein ligase RNF144A-A gene (LOC111254119), amplified on the DNA template of family 284 and 43.

650 **Table S1.** Similar rates of heterozygosity in males and females. Significant difference between
651 males and females in honey bee RNA and DNA, but no significant difference in Varroa mite
652 (Wilcoxon rank sum test with continuity correction)

Sex	Median	Mean	n	sd	Organism	Data	p-value
Female	0.48	0.48	30	0.02	Bee	DNA	0.00
Male	0.13	0.12	6	0.09	Bee	DNA	0.00
Female	0.47	0.47	7	0.02	Bee	RNA	0.00
Male	0.09	0.10	7	0.03	Bee	RNA	0.00
Female	0.18	0.20	93	0.12	Varroa	DNA	0.82
Male	0.20	0.20	56	0.14	Varroa	DNA	0.82
Female	0.85	0.86	5	0.03	Varroa	RNA	0.86
Male	0.90	0.90	2	0.08	Varroa	RNA	0.86

653

Table S2. Detailed information for each of the Varroa mites sequenced for the pedigree construction. All samples belong to Varroa destructor mite, collected from a Western honey bee, Apis mellifera liguistica sealed cell. honey bee hive located at OIST experimental apiary, Onna-son Okinawa, Japan. Samples were collected by Nurit Eliash from July to October 2020, and extracted from whole-body tissue by Endo Tetsuya at OIST.

Sample name	Biosample accession	Family	Developmental stage	Generation	Sex	Mapped reads	Mean coverage
110_110_fnd	SAMN24905483	110	Adult	0	female	98.22%	7.6
110_110a_son	SAMN24905484	110	Adult	1	male	98.73%	12.9
110_111_dat	SAMN24905485	110	Adult	1	female	99.27%	17.9
110_111a_grn	SAMN24905486	110	Nymph	2	not determined	99.06%	16.5
110_111b_grn	SAMN24905487	110	Egg	2	not determined	99.14%	16.1
110_111c_grnson	SAMN24905488	110	Adult	2	male	99.44%	24.7
110_111d_grndat	SAMN24905489	110	Adult	2	female	99.39%	20.7
110_111e_grn	SAMN24905490	110	Nymph	2	not determined	99.13%	16.4
110_111f_grn	SAMN24905491	110	Nymph	2	not determined	99.34%	14.5
133_133_fnd	SAMN24905492	133	Adult	0	female	99.48%	33.9
133_133a_son	SAMN24905493	133	Adult	1	male	99.49%	29.2
133_134_dat	SAMN24905494	133	Adult	1	female	99.35%	19.4
133_134a_grnson	SAMN24905495	133	Adult	2	male	98.99%	13.0
133_134b_grn	SAMN24905496	133	Nymph	2	not determined	99.40%	15.9
133_134c_grn	SAMN24905497	133	Egg	2	not determined	99.52%	19.1
177_177_fnd	SAMN24905498	177	Adult	0	female	99.55%	24.6
177_177a_son	SAMN24905499	177	Adult	1	male	99.44%	25.2
177_178_dat	SAMN24905500	177	Adult	1	female	99.53%	27.9
177_178a_grndat	SAMN24905501	177	Adult	2	female	98.46%	6.8
177_178b_grnson	SAMN24905502	177	Adult	2	male	98.60%	7.3
177_178c_grn	SAMN24905503	177	Nymph	2	not determined	99.02%	13.9
177_178d_grn	SAMN24905504	177	Nymph	2	not determined	98.93%	16.8
187_187_fnd	SAMN24905505	187	Adult	0	female	99.40%	17.8
187_187a_son	SAMN24905506	187	Adult	1	male	98.79%	11.2
187_188_dat	SAMN24905507	187	Adult	1	female	99.39%	25.1
187_188a_grnson	SAMN24905508	187	Adult	2	male	98.11%	7.5
187_188b_grn	SAMN24905509	187	Nymph	2	not determined	98.85%	11.4
187_188c_grn	SAMN24905510	187	Nymph	2	not determined	98.85%	10.1
240_240_fnd	SAMN24905511	240	Adult	0	female	99.33%	27.8
240_240a_son	SAMN24905512	240	Adult	1	male	99.20%	18.9
240_241_dat	SAMN24905513	240	Adult	1	female	99.54%	20.3
240_241a_grndat	SAMN24905514	240	Adult	2	female	99.45%	21.8
240_241b_grndat	SAMN24905515	240	Adult	2	female	98.66%	10.6
240_241c_grnson	SAMN24905516	240	Adult	2	male	98.81%	9.9
240_241d_grn	SAMN24905517	240	Nymph	2	not determined	99.35%	19.2
240_241e_grn	SAMN24905518	240	Nymph	2	not determined	98.39%	14.8
266_266_fnd	SAMN24905519	266	Adult	0	female	98.27%	7.5
266_266a_son	SAMN24905520	266	Adult	1	male	98.32%	6.5
266_267_dat	SAMN24905521	266	Adult	1	female	98.84%	20.4
266_267a_grndat	SAMN24905522	266	Adult	2	female	98.29%	16.3
266_267b_grndat	SAMN24905523	266	Adult	2	female	99.41%	24.7
266_267c_grn	SAMN24905524	266	Nymph	2	not determined	99.39%	32.4
284_284_fnd	SAMN24905525	284	Adult	0	female	99.46%	36.4
284_284a_son	SAMN24905526	284	Adult	1	male	99.22%	23.2
284_285_dat	SAMN24905527	284	Adult	1	female	99.27%	24.2
284_285a_grnson	SAMN24905528	284	Adult	2	male	99.30%	17.1
284_285b_grndat	SAMN24905529	284	Adult	2	female	99.47%	28.2
284_285c_grn	SAMN24905530	284	Nymph	2	not determined	99.39%	28.8
284_285d_sis	SAMN24905531	284	Adult	1	female	99.50%	37.0
300_300_fnd	SAMN24905532	300	Adult	0	female	99.00%	18.9
300_300a_son	SAMN24905533	300	Adult	1	male	99.37%	31.3
300_301_dat	SAMN24905534	300	Adult	1	female	98.01%	7.5
300_301a_grnson	SAMN24905535	300	Adult	2	male	98.05%	9.1
300_301b_grn	SAMN24905536	300	Nymph	2	not determined	99.52%	27.3
300_301c_grn	SAMN24905537	300	Egg	2	not determined	99.25%	20.6
302_302_fnd	SAMN24905538	302	Adult	0	female	99.16%	17.1
302_302a_son	SAMN24905539	302	Adult	1	male	99.22%	19.5
302_303_dat	SAMN24905540	302	Adult	1	female	99.36%	33.7
302_303a_grn	SAMN24905541	302	Nymph	2	not determined	99.37%	23.6

302_303b_grnson	SAMN24905542	302	Adult	2	male	98.94%	23.9
322_322_fnd	SAMN24905543	322	Adult	0	female	98.31%	7.7
322_322a_son	SAMN24905544	322	Adult	1	male	98.59%	16.6
322_323_dat	SAMN24905545	322	Adult	1	female	98.49%	10.8
322_323a_grndat	SAMN24905546	322	Adult	2	female	99.08%	16.0
322_323b_grnson	SAMN24905547	322	Adult	2	male	98.53%	11.1
322_323c_grn	SAMN24905548	322	Nymph	2	not determined	98.20%	9.4
322_323d_grn	SAMN24905549	322	Nymph	2	not determined	98.40%	9.6
338_338_fnd	SAMN24905550	338	Adult	0	female	98.34%	28.8
338_338a_son	SAMN24905551	338	Adult	1	male	99.14%	15.4
338_339_dat	SAMN24905552	338	Adult	1	female	99.48%	28.7
338_339a_grndat	SAMN24905553	338	Adult	2	female	99.48%	28.3
338_339b_grnson	SAMN24905554	338	Adult	2	male	98.92%	13.3
338_339c_grn	SAMN24905555	338	Nymph	2	not determined	99.11%	16.4
338_339d_grn	SAMN24905556	338	Nymph	2	not determined	99.40%	28.1
338_339e_grn	SAMN24905557	338	Egg	2	not determined	98.62%	13.3
400_400_fnd	SAMN24905558	400	Adult	0	female	99.25%	22.2
400_400a_son	SAMN24905559	400	Adult	1	male	99.22%	25.1
400_401_dat	SAMN24905560	400	Adult	1	female	99.13%	28.3
400_401a_grnson	SAMN24905561	400	Adult	2	male	99.31%	29.1
412_412_fnd	SAMN24905562	412	Adult	0	female	99.34%	22.2
412_412a_son	SAMN24905563	412	Adult	1	male	99.30%	25.7
412_413_dat	SAMN24905564	412	Adult	1	female	99.28%	19.9
412_413a_grnson	SAMN24905565	412	Adult	2	male	98.56%	12.2
412_413b_grn	SAMN24905566	412	Nymph	2	not determined	98.63%	13.9
426_426_fnd	SAMN24905567	426	Adult	0	female	99.08%	14.4
426_426a_son	SAMN24905568	426	Adult	1	male	98.73%	10.5
426_427_dat	SAMN24905569	426	Adult	1	female	99.43%	28.8
426_427a_grndat	SAMN24905570	426	Adult	2	female	98.82%	27.7
426_427b_grnson	SAMN24905571	426	Adult	2	male	98.89%	16.1
426_427c_grn	SAMN24905572	426	Nymph	2	not determined	99.35%	21.2
426_427d_grn	SAMN24905573	426	Nymph	2	not determined	99.30%	28.9
426_427e_grn	SAMN24905574	426	Nymph	2	not determined	99.20%	20.6
426_427f_grn	SAMN24905575	426	Nymph	2	not determined	99.50%	32.4
43_43_fnd	SAMN24905439	43	Adult	0	female	98.54%	8.2
43_43a_son	SAMN24905440	43	Adult	1	male	98.89%	13.1
43_43b_sis	SAMN24905441	43	Adult	1	female	99.21%	17.3
43_44_dat	SAMN24905442	43	Adult	1	female	99.44%	29.8
43_44a_grndat	SAMN24905443	43	Adult	2	female	99.54%	29.1
43_44b_grnson	SAMN24905444	43	Adult	2	male	99.12%	13.9
43_44c_grn	SAMN24905445	43	Nymph	2	not determined	98.94%	10.6
43_44d_grn	SAMN24905446	43	Nymph	2	not determined	99.37%	21.1
458_458_fnd	SAMN24905576	458	Adult	0	female	99.36%	24.4
458_458a_son	SAMN24905577	458	Adult	1	male	98.72%	12.2
458_459_dat	SAMN24905578	458	Adult	1	female	99.22%	27.9
458_459a_grnson	SAMN24905579	458	Adult	2	male	99.35%	31.3
458_459b_grn	SAMN24905580	458	Nymph	2	not determined	99.37%	31.9
458_459c_grn	SAMN24905581	458	Egg	2	not determined	99.35%	27.2
46_46_fnd	SAMN24905447	46	Adult	0	female	99.04%	12.8
46_46a_son	SAMN24905448	46	Adult	1	male	98.64%	19.6
46_46b_sis	SAMN24905449	46	Nymph	1	not determined	99.24%	14.8
46_47_dat	SAMN24905450	46	Adult	1	female	99.53%	28.6
46_47a_grn	SAMN24905451	46	Nymph	2	not determined	98.95%	10.1
46_47b_grn	SAMN24905452	46	Nymph	2	not determined	99.21%	17.4
46_47c_grndat	SAMN24905453	46	Adult	2	female	99.42%	31.2
46_47d_grnson	SAMN24905454	46	Adult	2	male	99.19%	18.9
46_47e_grn	SAMN24905455	46	Nymph	2	not determined	99.42%	25.2
46_47f_grn	SAMN24905456	46	Nymph	2	not determined	99.08%	14.8
46_47g_grn	SAMN24905457	46	Nymph	2	not determined	99.33%	21.3
476_476_fnd	SAMN24905582	476	Adult	0	female	98.98%	29.3
476_476a_son	SAMN24905583	476	Adult	1	male	98.66%	20.6

476_477_dat	SAMN24905584	476	Adult	1	female	98.24%	15.6
476_477a_grndat	SAMN24905585	476	Adult	2	female	98.85%	21.2
476_477b_grnson	SAMN24905586	476	Adult	2	male	98.87%	29.6
476_477c_grndat	SAMN24905587	476	Adult	2	female	98.83%	23.5
476_477d_grndat	SAMN24905588	476	Adult	2	female	98.92%	23.9
476_477e_grn	SAMN24905589	476	Egg	2	not determined	99.20%	29.0
478_478_fnd	SAMN24905590	478	Adult	0	female	99.08%	32.9
478_478a_son	SAMN24905591	478	Adult	1	male	98.78%	24.1
478_479-1_dat	SAMN24905592	478	Adult	1	female	99.03%	30.6
478_479-1a_grnson	SAMN24905593	478	Adult	2	male	98.58%	25.6
478_479-1b_grndat	SAMN24905594	478	Adult	2	female	98.27%	15.5
478_479-1c_grn	SAMN24905595	478	Nymph	2	not determined	97.97%	11.3
478_479-1d_grn	SAMN24905596	478	Nymph	2	not determined	98.72%	22.1
478_479-1e_grn	SAMN24905597	478	Egg	2	not determined	97.58%	9.1
478_479-2_dat	SAMN24905598	478	Adult	1	female	98.63%	15.3
478_479-2a_grndat	SAMN24905599	478	Adult	2	female	98.45%	9.1
478_479-2b_grnson	SAMN24905600	478	Adult	2	male	97.96%	9.3
478_479-2c_grn	SAMN24905601	478	Nymph	2	not determined	98.40%	5.6
478_479-2d_grndat	SAMN24905602	478	Adult	2	female	98.20%	11.0
48_48_fnd	SAMN24905458	48	Adult	0	female	99.09%	11.3
48_48a_son	SAMN24905459	48	Adult	1	male	99.33%	16.0
48_48b_sis	SAMN24905460	48	Nymph	1	not determined	99.06%	12.1
48_49_dat	SAMN24905461	48	Adult	1	female	99.47%	29.6
48_49a_grn	SAMN24905462	48	Nymph	2	not determined	99.46%	27.6
48_49b_grn	SAMN24905463	48	Nymph	2	not determined	99.39%	23.4
48_49c_grn	SAMN24905464	48	Egg	2	not determined	98.82%	9.8
48_49d_grn	SAMN24905465	48	Egg	2	not determined	98.56%	5.6
48_49e_grn	SAMN24905466	48	Nymph	2	not determined	98.95%	16.9
48_49f_grn	SAMN24905467	48	Nymph	2	not determined	98.83%	18.5
48_49g_grn	SAMN24905468	48	Egg	2	not determined	99.39%	27.0
498_498_fnd	SAMN24905603	498	Adult	0	female	97.82%	9.2
498_498a_son	SAMN24905604	498	Adult	1	male	97.32%	11.4
498_499_dat	SAMN24905605	498	Adult	1	female	98.98%	37.6
498_499a_grnson	SAMN24905606	498	Adult	2	male	98.81%	29.0
498_499b_grndat	SAMN24905607	498	Adult	2	female	98.02%	13.5
498_499c_grndat	SAMN24905608	498	Adult	2	female	98.55%	26.6
498_499d_grn	SAMN24905609	498	Nymph	2	not determined	98.12%	18.2
498_499e_grn	SAMN24905610	498	Egg	2	not determined	97.91%	15.7
502_502_fnd	SAMN24905611	502	Adult	0	female	97.75%	9.8
502_502a_son	SAMN24905612	502	Adult	1	male	98.02%	11.1
502_503_dat	SAMN24905613	502	Adult	1	female	98.08%	14.0
502_503a_grndat	SAMN24905614	502	Adult	2	female	98.04%	11.2
502_503b_grnson	SAMN24905615	502	Adult	2	male	98.32%	12.2
502_503c_grn	SAMN24905616	502	Nymph	2	not determined	99.05%	29.5
502_503d_grn	SAMN24905617	502	Nymph	2	not determined	98.22%	17.9
502_503e_grn	SAMN24905618	502	Egg	2	not determined	98.94%	30.2
502_503f_grndat	SAMN24905619	502	Adult	2	female	98.70%	20.8
520_520_fnd	SAMN24905620	520	Adult	0	female	98.74%	28.2
520_520a_son	SAMN24905621	520	Adult	1	male	98.08%	15.6
520_521_dat	SAMN24905622	520	Adult	1	female	98.11%	15.8
520_521a_grn	SAMN24905623	520	Nymph	2	not determined	98.47%	18.3
520_521b_grn	SAMN24905624	520	Egg	2	not determined	98.59%	20.9
520_521c_grn	SAMN24905625	520	Egg	2	not determined	98.46%	13.3
520_521d_grn	SAMN24905626	520	Egg	2	not determined	99.02%	34.7
520_521e_grn	SAMN24905627	520	Egg	2	not determined	98.74%	29.0
534_534_fnd	SAMN24905642	534	Adult	0	female	98.98%	18.2
534_534a_son	SAMN24905643	534	Adult	1	male	99.23%	17.1
534_535_1_sis	SAMN24905644	534	Adult	1	female	98.92%	15.4
534_535_2_dat	SAMN24905645	534	Adult	1	female	98.68%	13.2
534_535_2a_grndat	SAMN24905646	534	Adult	2	female	99.10%	16.8
534_535_2b_grn	SAMN24905647	534	Nymph	2	not determined	99.25%	18.9

688 **Table S3.** Primers used for the sequencing validation using Sanger-sequencing method

Accession	Gene name	chromosome	forward primer	Reverse primer	length (bp)
LOC111243621	alpha-mannosidase 2x-like	NW_019211455.1	TGAGATGTCAATGGCAGGCA	AGGAAGCAAATTCGGGGATGT	404
LOC111252938	5-oxoprolinase-like	NW_019211459.1	TCCGCATATGGACTTGCGTT	ATCCCAATTGCTCTGACCCG	374
LOC111254119	probable E3 ubiquitin-protein ligase RNF144A-A	NW_019211460.1	ACCAGATCGCACCCTTATGC	AGATGCTTTGCTATCCGCCC	245

689