Incipient species or social polymorphism?

Diversity in the desert ant *Cataglyphis*

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Abstract

Species are the fundamental units upon which evolutionary research is based. In insects, due to the high level of hybridization, the delimitation of such species can be challenging. The genus *Cataglyphis* presents a high level of diversification, making it an excellent model with which to study evolutionary paths. Israel appears to be a “hot spot” for recent speciation in this genus. Although previous studies have described multiple species of *Cataglyphis* in Israel, a recent genetic study has questioned the existence of some of these historically described species. The present study focuses on an apparent species complex that is distinguishable by its mitochondrial DNA (and therefore named mitotypes) but not by its nuclear DNA. Using a multi-method approach (genetics, chemistry and behavior), we show that these mitotypes also differ in their social structures and are readily distinguishable by their cuticular hydrocarbons profiles. While the different mitotypes are in general allopatric, at our study site they all coexist but nonetheless maintain the observed differences between them. This raises many evolutionary questions: Are these incipient species that have diverged with gene flow, or is this a case of social and chemical polymorphism that is maintained within a single species.

Key words: *Cataglyphis*, species delimitation, social polymorphism, cuticular hydrocarbons, polygyne, speciation

Introduction

Species are the fundamental units upon which evolutionary research is based. Although the biological species concept requires a reproductive barrier between species (Mayr, 2000), many alternative concepts (ecological, evolutionary, cohesion, phylogenetic, and others) that are based on different common characteristics within species, have been proposed (De Queiroz, 2007). Much has also been discussed about whether sympatric speciation can occur and what mechanisms might facilitate it (Bolnick and Fitzpatrick, 2007). Sympatric speciation assumes adaptive divergence of multiple traits in

order to generate strong ecologically-based reproductive isolation. However, the possibility of sympatric speciation without some gene flow does not seem probable and, indeed, recent studies have reported that speciation with gene flow is more common than previously thought (Nosil, 2008; Feder, Egan and Nosil, 2012). In insects, incomplete divergence in mating biology can lead to relatively high levels of hybridization between species, challenging the biological species concept and facilitating the possibility of cryptic speciation and speciation with gene flow (Cognate, Seybold and Sperling, 1999; Nosil, Harmon and Seehausen, 2009; Matsubayashi, Ohshima and Nosil, 2010). Generally, speciation can be considered as a continuum on which we can detect various transitions or diversifications such as morphological, genetic, and behavioral, which at different time points may have different levels of differentiation that might not yet show full evolutionary independence, thus named incomplete lineage sorting (Hey and Pinho, 2012; Fišer, Robinson and Malard, 2018). In order to accurately describe diversifications between closely-related species that might be on the species continuum, classical morphology-based taxonomy is not always sufficient, but requires additional methods of identification such as chemical, behavioral, ecological, and molecular (Seifert, 2009; Ross *et al.*, 2010; Kather and Martin, 2012; Ronque *et al.*, 2016; Fišer, Robinson and Malard, 2018). Differences between species, unlike between populations within a species, should embrace vast changes that cannot be explained as simple polymorphism. For example, cuticular hydrocarbon profiles, the presumed nestmate recognition cues in social insects, slightly differ between nests (exemplifying normal polymorphism) but greatly differ between species (exemplifying major changes). Likewise, behavioral changes that affect either mating preference or mating systems, or foraging, or habitat selection, can all lead to niche displacement and a drive for sympatric speciation (Arnqvist *et al.*, 2000). In ants, cases of intranidal mating, gyne (unmated ant queens) morphological polymorphism, a shift from monogyne (single queen) to polygyne (multiple queen) colonies, or host-parasite interactions were all suggested to result in sympatric speciation (Seifert, 2009; Torres *et al.*, 2018). This scenario leads to mating place separation and facilitates further genotype divergence,

eventually leading to novel genotypes and new species (Seifert, 2010). Social polymorphism (both monogyne and polygyne colonies in the same species and the same population) has been described in several ant species, including *Pheidole*, *Formica*, *Leptothorax*, etc. (Bourke and Franks, 1995). Finally, recent studies have shown that there are few cases in which social polymorphism and other traits do not lead to speciation but are acquired by means of a ‘super- gene’, also named ‘social chromosome’ distributed in the population (Wang *et al.*, 2013; Purcell *et al.*, 2014; Taylor and Campagna, 2016). However, this ‘social chromosome’ mechanism is rather rare and has been described only twice, while most incidents of social polymorphism are mainly believed to depend on ecological constraints.

The ant genus *Cataglyphis* comprises over a hundred species (Agosti, 1990) displaying various social and population structures, ranging from the basic monogynous multicolonial population to polygynous supercolonies, making it an excellent model to study evolutionary paths (Lenoir *et al.*, 2009; Boulay *et al.*, 2017; Timmermans *et al.*, 2010; Leniaud *et al.*, 2011; Jowers *et al.*, 2013). The genus also expresses various reproductive strategies, from sexual to the asexual (parthenogenetic) reproduction of new reproductive queens (Leniaud *et al.*, 2012; Eyer *et al.*, 2013; Darras, Kuhn and Aron, 2014; Aron, Mardulyn and Leniaud, 2016). Israel appears to be a “hot spot” for recent speciation in this genus: in the *bicolor* group, we identified eight mitochondrial types (Eyer *et al.*, 2017), in the *albicans* group there are nine identified mitochondrial types (Eyer and Hefetz, 2018). These mitochondrial types do not always concur with nuclear delimitation methods, and it is not clear whether mitochondrial types that have been previously used to delimit species can also serve as identifiers in this case of the *bicolor* group.

Here, we examined an apparent species complex of the previously described putative species *C. niger* and *C. savignyi* (Agosti, 1990; Radchenko, 2001), and a population tentatively identified as *C. drusus* (henceforth termed the “niger complex”). In a previous study, these putative species were found to exhibit different mitochondrial haplotypes (hereafter called mitotypes), but could not be differentiated

on the basis of the nuclear genes used (Eyer *et al.*, 2017). To disentangle this species complex, we used further genetic tools (ddRAD sequencing and microsatellite markers), together with the chemistry of cuticular hydrocarbons, and behavioral assays designed to examine colony insularity.

Methods

Sample collection:

Sample collection was divided according to the different analyses methods: 1. Samples collected from different geographic areas across Israel for the RAD sequencing analysis 2. samples collected from the Betzet and the Tel-Baruch populations for the microsatellite and the CHC analyses; 3. samples collected from the Betzet and the Tel-Baruch populations for behavioral experiments; and 4. samples of queens, newly mated gynes, and their spermatheca contents for mitotype analysis from the Tel-Baruch population.

1. 118 individual were collected from 55 locations across Israel, and from Turkey (kindly collected by Dr. Kadri Kiran, Trakya University, Turkey), 76 were originally collected along with samples for Eyer *et al.* 2017 and the rest 42 samples were newly collected for this project (sample collections are detailed in S1). All samples for genetic analysis were kept in absolute ethanol until extraction.

2. Nests were collected from two coastlines locations: Betzet (33° 4'36.09"N, 35° 6'32.05"E) and Tel Baruch (32° 7'33.76"N, 34°47'9.04"E). These locations present similar ecological niches that are characterized by semi-stabilized sand dunes. We sampled all visible nests in a plot of 200*200 m, as well as a transect of 1 km (at 100 m intervals). From the Betzet and Tel Baruch sites respectively, we collected partial nests-queenless nests (QL; n= 57/39), and whole nests/queenright nests (QR; n= 2/11; at). All the nests in the experimental plot were excavated over four consecutive days to avoid pseudo-

replication due to nest migration. Ants were dissected as follows: the head, for CHC content, was kept in hexane; the thorax and legs, for DNA extraction, were kept in 100% ethanol; and the abdomen was frozen at -20°C and kept for future analysis. Table S2 details the number of nests used in each analysis and collection site.

3. Nests at the above-mentioned locations were thoroughly excavated to include the queen, all workers, and brood. To ensure that the entire nest was collected, excavation was further extended by an extra 0.5-1 m in all directions after having observed the last gallery. In the Betzet location, despite our thorough excavations the queens were difficult to locate, so we used both QR (n=3) and QL nests (n=15) for the experiments. At Tel Baruch we successfully excavated and used 10 QR nests.

4. Established queens were collected from the above excavated nests. Newly mated gynes were collected from the Tel Baruch population during the 21 consecutive days of nuptial flight. Some of the gynes had shed their wings and some had also begun to dig a new nest. All were dissected and their spermatheca content was extracted. Sperm was kept in Ringer's buffer and frozen at -80°C until DNA extraction.

Genetic analysis:

Mitochondrial haplotypes - were determined by genotyping Cytochrom B using one worker per nest (Eyer et al., 2017). CytB primers used: CB1 (Forward) TATGTACTACCATGAGGACAAATATC and CB2 (Reverse) ATTACACCTCCTAATTTATTAGGAAT, annealing temperature was 48°C (Simon *et al.*, 1994). PCR products were purified with ExoSAP-IT™ PCR purification kit (Applied Biosystems). Sequencing was performed on an ABI 3730 Genetic Analyzer (Applied Biosystems). Base calling and sequence reconciliation were inspected by eye and performed using Unipro UGENE v 1.3. Sequences were aligned using MUSCLE algorithm (Edgar, 2004) implemented in UGENE Aligner, together with sequences previously used in Eyer 2017.

RAD sequencing-

We held suspicions that the genetic markers employed in Eyer *et al.* 2017 were possibly not sensitive enough to detect differences between the mitotypes so we decided to use Double Digest Restriction Associated DNA (**ddRAD**) Sequencing that provides many more genomic markers enabling better accuracy for delimiting species. All the DNA for this analysis was extracted using Qiagen blood and tissue DNA extraction kit following the standard protocol. We used a modified protocol of (Parchman *et al.*, 2012; Peterson *et al.*, 2012), to construct double-digest RAD libraries, modifications added by A. Brelsford, A. Mastretta-Yanes, J. Leuenberger, R. Sermier. Changes were as follows: Added dual-index barcoding to allow multiplexing >96 samples per library. The Y-adapter for MseI from Peterson et al (ddRAD) was used to prevent amplification of MseI-MseI fragments. Simplified restriction and ligation mixes using CutSmart buffer. Purification post-ligation was done using Agencourt AMPure beads. PCR using Q5 Hot Start Polymerase (New England BioLabs). PCR modified to decrease number of cycles by increasing number of replicates and starting DNA volume. Addition of primers and dNTPs for a final thermal cycle, in order to reduce production of single-stranded or heteroduplex PCR products. Final cleanup and size selection was carried out with AMPure beads. Three samples were omitted from the final results due to poor sequencing yield. All samples were multiplexed and sequenced by single-end 100bp reads on 2 lanes of an Illumina HiSeq4000 sequencer.

Processing of RAD sequence data- A total of 648,303,541 reads were sequenced, with an average of 5,026,520 reads per sample (275,586 reads for the lowest coverage sample, pol633_u). Dividing the number of reads by the 67,687 EcoRI restriction sites in the reference genome, it is an average coverage of 37X. The raw reads were initially processed using the *Stacks* pipeline (Catchen *et al.*, 2011; Catchen, 2013) and low quality reads were discarded. These were defined as reads in which the score drops below an averaged phred score of 10 in a sliding window of 15% of the reads' length, that is 14 bases for

a read length of 93 bases. After this initial processing, 583,275,943 reads that were mapped to a sample remain (92%). A total of 459,081,529 reads were mapped to the reference genome of *C. niger* (version Cnig_gn1) using *Bowtie2* (Alexander, Novembre and Lange, 2009). We filtered the alignments allowing for a maximum of four mismatches. To filter non-unique mappings, we removed alignments where the second best hit had less than twice the number of mismatches found in the best hit. After this filtering, 369,719,665 alignments remained. At this point, the actual coverage of mapped loci was calculated, with an average of 22X. We continued to analyze the mapped sequences to identify single nucleotide polymorphic sites (SNPs) using the *Stacks* pipeline and created a catalog containing 300,509 SNPs for the 126 samples.

STRUCTURE analysis for RAD samples- Individual samples were divided to seven populations according to their mitotype: *israelensis*, *drusus*, *niger*, *savignyi*, *holgerseni*, *isis* from Israel, and from Turkey- *noda*. The data in the catalogue was filtered as follows: 1) loci were removed if they had more than 7% missing data (samples where a genotype could not be called at that locus). 2) Minimal minor allele frequency (MAF) of 1% (a minimum of 3 allele copies in the 230 chromosomes of 115 diploid individuals). 3) A minimum of five reads per individual for calling a genotype. Each sample was required to have genotype calls in at least 80% of the loci. This resulted in a catalogue of 19,995 high confidence SNPs that were used in the population structure analysis (supplementary Fig. S1).

STRUCTURE (Pritchard, Stephens and Donnelly, 2000; Falush, Stephens and Pritchard, 2007) was run over *K* (number of expected clusters) values of 3 – 10; for each *K* value, 100 iterations of *STRUCTURE* were run, each over a different set of 1,000 SNPs randomly chosen out of the high confidence SNPs. In each set, a minimal genomic distance of 2,000 nucleotides between sites was maintained to comply with *STRUCTURE*'s assumption of no linkage disequilibrium. The program was run with 1,100,000 MCMC iterations, and the first 100,000 were discarded (burn-in period). All the other

parameters were kept at default. The results were analyzed using *CLUMPAK* (Kopelman *et al.*, 2015) and combined for each K value.

To test whether the three species in the complex are genetically separated or not we used full maximum likelihood calculations to compare between two multi-species coalescent models as implemented by 3s program (Yang, 2002; Zhu and Yang, 2012; Dalquen, Zhu and Yang, 2017). The null model which does not allow gene flow between two closely related species and an alternative model that does. Both models include a third species that is used as an out-group. This species is assumed not to have genetic flow with the species tested. We performed multiple such likelihood ratio tests (LRT) between different combinations of individuals of the populations of *C. savignyi*, *C. drusus*, *C. niger*, *C. israelensis* and *C. isis*, first to check for gene flow between *C. israelensis* and any one of the species in the complex with the out-group of *C. isis* and then between the species of the complex with the out-group of *C. israelensis*.

93 bases long sequences of these individuals, product of the RAD sequencing, were aligned to a reference genome of *C. drusus* as before, (allowing a maximum of 4 mismatches for the best hit and a minimum of twice as many mismatches as the best hit for the second best hit), only this time no gaps were allowed. Based on the position of the sequences in relation to the reference genome, 3 sequences from 3 different individuals were aligned together in one of the following configuration: 123 – a sequence from individual of species 1, a sequence from an individual of species 2 and a sequence from individual of species 3; 223 – two sequences from two different individuals of species 2 and a sequence from individual of species 3; and similarly 113. This is assuming species 1 and 2 are tested for gene flow while species 3 is used as an out-group. At least 2000 bases gap was maintained between the triplets of aligned sequences (as inferred from the reference genome). For each locus one of the three possible alignment configurations was chosen at random.

For each combination of species, 3s was run three times over the corresponding aligned sequences, using the Gaussian quadrature number of points = 16 and different seed values. Then LRT was performed between the highest scoring null and alternative models. The null model of no genetic flow was rejected if $2D_{lnL} > 5.99$ (corresponding to χ^2 distribution with two degrees of freedom and significance level of 0.05 for type I mistake).

Microsatellites – To understand the genetic population structure as well as the social structure we used a microsatellite markers analysis. The analysis was performed on 648 workers of the Betzet population (*C. drusus* only; $X \pm SE = 11.42 \pm 0.1$, N=57 nests). DNA was extracted with 5% CHELEX (BIO-RAD) and then amplified with 11 microsatellite markers (previously designed for: *C. hispanica* - Ch01, Ch08, Ch10, Ch11, Ch12; Darras, Kuhn and Aron, 2014, *C. cursor* - Cc54, Cc65, Cc96 and Cc11; Pearcy *et al.*, 2004, and *C. niger* - Cn02, Cn04; Saar *et al.*, 2014). Similarly, genetic analysis was performed on 548 workers of the Tel Baruch population ($X \pm SE = 21.08 \pm 0.45$, N=26 nests). DNA was amplified with 7 microsatellite markers (previously designed for *C. hispanica* - Ch23; Darras *et al.* 2014, *C. cursor* - Cc99, Cc54, Cc51; Pearcy *et al.* 2004, and *C. niger* - Cn02, Cn04, Cn08; Saar *et al.* 2014). Amplification was done using Type-it PCR mix (QIAGEN). PCR products were sequenced with ABI3500, genotypes were then analyzed with GeneMarker. Microsatellite data were checked for linkage disequilibrium, fixation index, and G-test using GENEPOP (Raymond and Rousset, 1995), heterozygosity was checked using both GENEPOP and FSTAT (Goudet, 1995), and F_{st} was checked using FSTAT. To assess the number of queens, queen genotypes, and the number of matings we used the program COLONY (Jones and Wang, 2010). The program STRUCTURE (Pritchard, Stephens and Donnelly, 2000) was used to evaluate the number of colonies in the population. The number of K (number of possible colonies, from 1 to n size) was evaluated by the Evanno method using STRUCTURE Harvester (Earl, 2012). PCA of microsatellite data

was performed as described by Ryan *et al.*, 2017 (using R version 3.3.3; library *adeigenet* and *ggplot2* for visualization), and relatedness was estimated using Coancestry (Wang, 2011; Queller GT method).

Behavioral assays:

In order to examine nest insularity and colony composition at the population level we used nest-merging experiments (Reiner Brodetzki & Hefetz 2018). We composed queenright (QR) colonies (n=3 and n=10 for Betzet and Tel Baruch, respectively) comprising approximately 200 workers maintained in nesting boxes prior to the experiment, as well as freshly excavated queenless (QL) nests (n=15). Workers and queens of each colony were marked on the thorax with different colors of nail polish, and then left for a day to get acclimate. At the onset of the merging experiment the two nests were connected to a common foraging arena (size 20x20 cm). Sand and food were placed in the foraging arena and water and cotton balls in the enclosed nests. Scan observations were performed for up to three days (merging was stopped on two occasions in order to save the queens), while recording interactions between non-nestmates, including aggression, trophallaxis, adult carrying, and antennation. For the Betzet population, merging experiments were carried out between 18 nests, three of which were QR and 15 were QL. The merging tests between the QL nests were performed up to a week after excavation, in order to avoid a possible 'QL nest behavior', in which nests that are rendered queenless have a tendency to lose their specific nest 'signature' over time. All nests were tested against all nests. If nests merged together, they were left as one colony. If we observed over ten aggressive interactions within ten minutes, we stopped the test and separated the two nests. In Tel Baruch merging was done using 10 QR nests of the different mitotypes, as detailed in Table 4. If nests merged, they were left together as one colony with multiple queens.

Cuticular hydrocarbon analysis:

In order to distinguish colony boundaries according to their cuticular hydrocarbon profiles, we immersed individual heads in hexane (5-10 ants from each nest), containing 100 ng/μl of eicosane (C₂₀) as internal standard. Initial analysis was conducted by gas chromatography/mass spectrometry (GC/MS), using a VF-5ms capillary column that was temperature-programmed from 60°C to 300°C (with 1 min initial hold) at a rate of 10°C per min, with a final hold of 15 min. Compound identification was achieved according to their fragmentation pattern and respective retention indices compared to authentic compounds. We identified 74 and 60 compounds for the Tel Baruch and Betzet population samples respectively (supplementary Fig. S2 A+B). Quantitative analyses were performed by flame ionization gas chromatography (GC/FID), using the above running conditions. Peak integration was performed using the program Galaxie Varian 1.9. A total of 37/35 hydrocarbons were used in the Tel Baruch and Betzet analysis respectively (highlighted in bold in supplementary Fig. S2 A+B). Hydrocarbon profile specificity was determined using multivariate statistics, i.e., discriminant analysis using the stepwise forward mode. This module of the program works initially as a principle component analysis for reducing the number of variables to fit the number of cases, and then performs the discriminant function. We then used k-means clustering in order to determine the structuring of the population, and assessed the most probable K using kmeans function in *stats*, as well as mclust in *mclust* and nbclust in *NbClust* libraries in R. Plot was done by clusplot function in *cluster* library.

Results

RAD sequencing:

In order to clarify further the taxonomic status and species delimitation of the *bicolor* group in Israel (see Eyer *et al.*, 2017) we performed RAD sequencing on samples from different localities, which were

further divided into groups based on their mitotype *C. drusus* (n=9), *C. niger* (n=14), *C. savignyi* (n=47), *C. israelensis* (n=35), *C. isis* (n=4), *C. holgerseni* (n=3), and samples from Turkey (n=6) identified as *C. nodus*. *STRUCTURE* analysis for the possible K clusters was examined between 3 to 10 numbers of K's, resulting in the most probable Ks of 5, 6, and 7 (Figure 1). There was a clear separation between *C. isis*, *C. holgerseni* and *C. israelensis* at the 3 possible Ks, and in k=7 also *C. nodus* was clearly distinct. However, at K=6 or 7) distinction between the *C. holgerseni* and *C. nodus* samples was not clear cut (green or red and green respectively). In the *C. israelensis* population, some individuals showed genetic polymorphism that suggests hybridization between *C. israelensis* and the *C. niger complex*, mainly within the *savignyi* mitotype. *STRUCTURE* analysis for any attempted K (between 3 to 10) could not separate the three mitotypes populations of *C. drusus*, *C. niger* and *C. savignyi* and they are all clustered together (Fig. 1; colored in orange).

Species delimitations within the *niger* complex were put to the test using the maximum likelihood approach as implemented by 3s program, comparing a coalescent null model that does not allow for gene flow between tested species and an alternative model that does. At first *C. israelensis* was tested against each of the species in the complex with the out-group of *C. isis*. As the null model could not be rejected for any of the tests, we used *C. israelensis* as the out-group in the following tests to check for gene flow within the *niger* complex. In all three tests the null model was rejected and the *savignyi*, *niger* and *drusus* mitotypes were found to have gene flow between them. For the combination of *C. Holgerseni*, *C. savignyi* and *C. isis*, *C. Holgerseni* was found to have gene flow with the *savignyi* mitotype (Table 1).

The Betzet population

Genetic analysis

We examined a subset of 25 nests from the Betzet population for their mitotypes, and found that all of them presented the same mitotype, similar to the *drusus* group described in Eyer et al. (2017).

Microsatellite DNA analyses revealed that out of the 11 markers amplified and sequenced, three (Cc65, Cc96 and Cc11) were at linkage disequilibrium and therefore omitted from the analysis. The number of alleles in the remaining eight microsatellites loci ranged from 4 to 16. Mean heterozygosity was $H_o = 0.65$ (range 0.31-0.84). The fixation index F_{it} was slightly higher and significantly different from zero (mean $\pm$ SE jackknife = 0.09 ± 0.06 ; Z test = 11 p-value < 0.0001), indicating deviation from Hardy-Weinberg equilibrium and suggesting that mating was not completely random in the population studied.

F_{st} across the 59 nests sampled at Betzet was 0.19, while within nest relatedness was 0.35 (SE jackknife: 0.02). The G-test showed a result significantly different from zero between some of the nests, indicating that they were genetically differentiated and belonged to distinct colonies (21 nests), while for other nests the G test was not significantly different from zero and they are considered to belong to the same colonies (36 nests divided into 13 colonies). STRUCTURE analysis indicated that the population consists in 2, 3, or 13 genetic colonies (most probable K numbers; Fig.2 A-B) out of 57 nests analyzed. However, this result of 13 colonies did not seem very probable due to the dispersed location within the plot of the nests assigned to a particular genetic colony, and the fact that mixed genotypes were present within a single nest. K = 2 did not seem very probable either, as it suggests a unicolonial population (we did not see any evidence for such a population structure when observing the population in the field). We therefore conducted a Principle Component Analysis using the genetic colonies assigned by STRUCTURE division of K = 3, which revealed only a very small differentiation in genotypes, which were mainly very similar (PC1 = 3.4%, PC2 = 3.1%; Fig.2C).

Genotyping of workers from 42 nests showed that within each nest all of the workers' genotypes matched to a single queen; this conclusion was also supported by COLONY, unequivocally confirming monogyny. In the remaining 15 nests some of the worker genotypes (average of 1.7 workers per nest; SE= 0.22) did not perfectly match the queen's assigned genotype, suggesting either multiple queens (which is possible but less likely in light of the above data indicating monogyny), queen turn-over with workers that had remained in the nest from previous residing queens or the presence of drifting workers (workers who mistakenly enter and remain in another colony; a phenomenon documented in other *Cataglyphis* species; M. Knaden, personal communications). Comparing the reconstructed matriline of different nests based on the COLONY results, we assumed that nests with similar matriline belong to the same colony, resulting in several polydomous colonies found in the population (Table S3 and in Fig. 2D). Considering monogyny, the COLONY analysis suggested polyandry with an average of 5.3 (SE= 0.22) males contributing to worker production (range: 2-9 males).

Cuticular hydrocarbons

Cuticular washes contained 60 identified hydrocarbons, of which 35 were used in the following analyses (supplementary Fig. S2A). CHC profiles were analyzed by calculating the relative amounts of each compound (peaks comprising inseparable compounds were considered as a single compound), for 49 nests (275 individuals). We then used the k-means approach to determine the level of structuring in the samples. The most probable number of K's was 4 according to all three analyses methods used (k means, mclust, and nbclust), with the two components explaining 70.93% of the variability (Fig.2F). We further performed a discriminant analysis based on the CHC profiles of the genetic colonies assigned by 'STRUCTURE' at K= 3. Colony assignment of three explains a high percentage of the variance (function1- 80.7% and function 2-19.3%), although the profiles are very similar and overlap (Fig.2E).

Behavior

A possible resolution of the presumed discrepancy between CHC and genetic data lies in performing behavioral assays, that is, in examining whether two distinct nests will merge to a single colony when given the opportunity. Out of the various 27 trials performed, 14 nest-pairs successfully merged, culminating in seven colonies, each encompassing one to five nests (Table 2 and Fig. 3). During the successful merges there was no aggression at all, either between workers or between workers and queens. The remaining 13 trials resulted in mutual aggression between workers that belonged to different nests, as well as aggression towards the queens (in QR nests). These trials were stopped after ten minutes to avoid further injuries to workers and queens. When merging occurred it was between either a QR and a QL nest or two QL nests, and generally when nests were located in close proximity (except for one case), suggesting that colonies with the *C. drusus* mitotype in the Betzet population are generally polydomous, having one central QR nest and several satellite QL nests.

Tel Baruch Population:

Genetic analysis

Genetic analysis (N= 26 nests) using CytB identified representatives of all three mitotypes belonging to the 'niger complex' in the sampled plot (*niger*, *savignyi* and *drusus*; n= 6, 17 and 3 nests, respectively).

Microsatellite DNA analysis was performed on 548 workers of the Tel Baruch population (mean number of workers typed per nest $\pm$ SE= 21.08 ± 0.45 , N= 26 nests) at 7 polymorphic loci. First, we analyzed all the nests in the population, irrespective of their mitotypes, assuming that they comprise a single population. The number of alleles ranged from five to 25. Mean heterozygosity was $H_o = 0.69$ (range: 0.4-0.87). The fixation index F_{it} was low, even though slightly higher than zero (mean $\pm$ SE jackknife= 0.02 ± 0.01) indicating that random mating predominates in the tested population. F_{st} was low at 0.12 while the within-nest relatedness was 0.23 (SE jackknife: 0.01). STRUCTURE indicated that the 26 nests analyzed consisted in 2, 3, or 6 colonies (Fig. 4A+B). Comparing colony distribution according to the

above results (Fig. 4C) with that of the mitotype distribution (Fig. 4D) showed incongruence. Furthermore, PCA distribution of the genotypes showed that, overall, they were very similar throughout the population (Fig. 4E).

Genetic analysis of the population structure considering the mitotypes separately gave a slightly different picture. Each mitotype had its distinct characteristics (Table 3); the *niger* mitotype had a very low F_{st} and very low within-nest relatedness, as predicted for polygyne colonies. The *drusus* and *savignyi* mitotypes, on the other hand, exhibited higher F_{st} and relatedness values, as predicted for monogyne colonies. However, perhaps due to the rather small sample size or the unique situation of this sympatric population, we obtained slightly different results to those for the other studied populations containing a single mitotype (data from: the Betzet population- only *drusus* mitotype in this paper, the Nitzanim population- only *niger* mitotype from Leniaud *et al.*, 2011, and the Rishon and Arad populations- only *savignyi* mitotype from Leniaud *et al.*, 2011; Saar *et al.*, 2014; Table 3).

Cuticular hydrocarbon analyses

For chemical analyses, we used head extracts of 76 ants belonging to 28 nests. We identified 74 compounds in the cuticular washes, of which 37 were used in the following analysis (supplementary Fig. S2B). Profile determination was constructed by calculating the relative amounts of each of the hydrocarbon components, and profile comparison was done by discriminant analysis, while grouping the ants according to the CytB mitotype. As can be seen in Fig.4F, there was a clear discrimination of CHC profiles among the three mitotypes.

Behavioral assay

Two alternative hypotheses emerged from the genetics data regarding the social structure of the Tel Baruch population: a single, highly polymorphic species, as suggested by the nuclear gene analyses; or

multiple species, as suggested by the mtDNA and CHC analyses. We therefore attempted to differentiate between the two hypotheses using behavioral assays comprising the possible merging of two distinct nests. Seven merging tests were performed using various pairs of nests (Fig. 5 and Table 4). First, we used nests of the *niger* mitotype located at distances between 100 and 500 meters from one another. In all cases the nests merged after a few hours, during which we observed both trophallaxis and mutual carrying between workers from the different nests. After 24 hours, the nests had fully merged, with the queens and brood having been moved into one of the nest boxes. All other pairing combinations, i.e., *drusus-niger*, *drusus-savignyi*, *savignyi-savignyi*, and *savignyi-niger*, resulted in aggressive behavior between the workers and none of these nests merged (Fig. 5).

Sperm analysis:

To examine whether assortative mating between the different mitotypes occurs, we genotyped, using CytB, the sperm extracted from the spermatheca of ten queens of the *niger* mitotype. Nine out of the ten sperm samples matched the *savignyi* mitotype and one matched the *niger* mitotype (Fig. 6). In some of the sequences there appeared to be more than one possibility of a nucleotide in certain positions, possibly indicating multiple sequences/mitotypes. This suggests that multiple males of multiple mitotypes inseminated a specific queen.

Discussion

A recent phylogeographic study of species delimitation in the *bicolor* group of the genus *Cataglyphis* in Israel (Eyer *et al.*, 2017) pointed out discrepancies between mitochondrial DNA and nuclear DNA sequences within the putative species *C. niger*, *C. savignyi*, and *C. drusus*, i.e., the *C. niger* complex. The three mitotypes also differ in their social structure. The *savignyi* mitotype is monogyne demonstrating either monodomous (Leniaud *et al.*, 2011) or polydomous (Saar *et al.*, 2014; mistakenly identified as *niger* when published) nesting behavior; whereas the *niger* mitotype is polygyne and is organized as a

supercolony (Leniaud *et al.*, 2011). The social and population structure of the *drusus* mitotype has not previously been investigated. Furthermore, this study revealed that all three mitotypes occur sympatrically in the Tel Baruch site. Therefore, disentangling the species identity of this complex is expected to provide insight not only into the process of sympatric speciation, but also into the evolution of advanced social structures in ants.

First, we sought to rule out the possibility that the amplicons of the nuclear genes employed in Eyer *et al.* (2017) were not sensitive enough to detect differences between the three mitotype. We therefore conducted RAD sequencing on representatives of the above complex, along with samples from most of the locations described in Eyer *et al.* (2017), comprising other species, namely *C. holgerseni*, *C. isis*, *C. israelensis* and *C. nodus* (collected in Turkey). The RAD results were compatible with the nDNA data of the Eyer *et al.*, (2017) study, and ruling out the possibility of errors due to the limited sequence information for the study. *STRUCTURE* results confirmed the existence of five bicolor species in Israel, and most importantly confirmed the existence of gene flow between the three mitotypes of the *C. niger* complex (i.e. *niger*, *savignyi*, and *drusus*), suggesting that they constitute a single species. The results also indicate hybrid zones between *C. israelensis* and the *savignyi* mitotype of the *C. niger* complex.

The case of the *C. niger* complex is interesting and somewhat enigmatic. The three mitotypes have a peculiar distribution along the coastal plains of Israel (Eyer *et al.*, 2017). The northern part, from the Lebanese border to Mount Carmel, is inhabited exclusively by the *drusus* mitotype. To the south of Mount Carmel, the *savignyi* mitotype predominates, except for a patchy distribution of another species, *C. israelensis* (Ionesco and Eyer, 2016; Eyer *et al.*, 2017). Curiously, at the border of their distribution, we found hybrids between *C. israelensis* and the *savignyi* mitotype of the *C. niger* complex. The *savignyi* mitotype has the largest distribution. It occupies a large part of the sandy areas along the coast and it dominates in areas of heavier soil, extending to the more desert areas of Israel. The *niger* mitotype, in

contrast, is present only in the central coastal plain, where it has a patchy occurrence. Thus, at least in a large proportion of their distribution, although the *drusus* and *savignyi* mitotypes are allopatric, the nuclear sequencing data indicates free gene flow. This raises the question of whether we are encountering 3 incipient species that demonstrates rapid mitochondrial evolution but slower nuclear evolution; or a single species with long-range dispersal of sexuals (mostly through males because *Cataglyphis* females are generally known to have lower flying capability; Peeters and Aron, 2017), to account for the apparent gene flow among the highly allopatric populations.

The sympatric cooccurrence of all three mitotypes in the Tel Baruch population enables us to investigate the differences among the complex mitotypes within a natural setting without a confounding ecological variation. The microsatellite analysis reveals an *Fst* value of 0.12 across all the nests of the Tel-Baruch population, suggesting some level of diversification in the highly polymorphic markers (Wondji, Simard and Fontenille, 2002; Leppänen *et al.*, 2015). However, this estimate might not be accurate because some nests belong to the same colony. As reported previously, the *niger* mitotype is polygyne and supercolonial (Leniaud *et al.*, 2011). Here, we further established that this is an exclusively *niger* mitotype characteristic. Both *savignyi* and *drusus* at the study sites were monogyne and facultatively polydomous. The three mitotypes inhabiting the same plot were also distinctively different in their CHC composition. CHC's are the main mechanism through which ants maintain nest insularity (Soroker *et al.*, 1994; Hefetz, 2007; Kidokoro-Kobayashi *et al.*, 2012), thus making it highly important in such a diverse population. Within each of the mitotypes, differences in CHC profiles were rather small, consistent with populations exhibiting limited dispersal. Thus, the question of whether we have 3 incipient species (as indicated by morphology (Agosti, 1990), mitochondrial DNA, chemistry, and behavioral data), or a single species (as indicated by the nDNA genotyping) that is highly polymorphic (morphologically, chemically and to a certain degree genetically), remains open. While this does not pose a problem regarding

mitotype stability at the site (being maternally inherited), how the social structure (i.e., polygyny and supercoloniality) is maintained remains elusive.

According to the incipient species hypothesis, we expected assortative mating according to the different mitotypes. However, sperm analysis of mated gynes of the *niger* mitotype revealed a high presence of the *savignyi* mitotype (matching its abundance at the site), indicating the absence of mitotype-associated assortative mating. Such non-assortative mating can be the result of either recent admixture of the 3 species due to anthropogenic-associated geographic barrier destruction, leading the 3 species to merge into a single species, or supporting the single species hypothesis having 3 mitochondrial lineages, each of which associated with different social structure. Cross-mating between lineages within a species has been described in *Pogonomyrmex*, resulting in caste differentiation: eggs fertilized by males of the queen's lineage develop into new queens, while eggs fertilized by males of the opposite lineage develop into sterile workers (Schwander, Keller and Cahan, 2007). Another report of cross-mating between two social forms is that for *Solenopsis invicta* (Shoemaker and Ross, 1996; Ross and Shoemaker, 1997), in which polygyne queens mate with males from monogyne colonies.. In *Cataglyphis*, several species of the closely-related *C. altisquamis* group exhibit cross-mating between two hybridogenetic lineages, which facilitates worker production and genetic diversity (*C. velox*, *C. altisquamis*, *C. hispanica* and *C. mauritanica* Leniaud *et al.*, 2012; Aron, Mardulyn and Leniaud, 2016; Boulay *et al.*, 2017a). Here we showed that the polygyne *niger* queens partake in nuptial flights, which enables mating with males of the *savignyi* mitotype. However, since within-colony genetic diversity is satisfied in the case of non-related co-inhabiting queens (Reiner Brodetzki *et al.*, 2018), it is not clear whether there is a genetic necessity for cross-mitotype mating, paving the way for the evolution of assortative mating among mitotypes. In other *Cataglyphis* polygyne species, gyne morphology reveals the loss of wing muscles (Peeters and Aron, 2017), confining the queens to mating near or within the nest. Such a change in morphology would lead to mating place separation and sympatric speciation.

Another possible mechanism that could explain the social polymorphism exhibited by the *niger* complex, if considering the complex as a single species, is the occurrence of a non-combining ‘social chromosome’, as described in two other ant species, *Solenopsis invicta* and *Formica selysi* (Wang *et al.*, 2013; Purcell *et al.*, 2014). The independent evolution of a ‘social-chromosome’ mechanism may suggest that such a mechanism may also have evolved in *Cataglyphis*. However, the apparent differences in the mitochondrial DNA may indicate a maternal inheritance of these social traits as well, which raises the possibility of alternative explanations, not previously described in ants, such as a maternal genetic imprinting on social traits.

When considering possible mechanisms and road maps for sympatric speciation, we assume traits evolving in a single population in opposite directions. These traits should have major life-history effects that would drive for assortative mating, stopping gene flow and promoting speciation. Social behavior and recognition cues are such traits. The *C. niger complex* exemplifies a population in which some major traits have diverged while others have not, raising the possibility of a sympatric speciation continuum based on a differentiation in sociality.

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References

499 Agosti, D. (1990) 'Review and reclassification of *Cataglyphis* (Hymenoptera, Formicidae)', *Journal of*
500 *Natural history*. Taylor & Francis Group, 24(6), pp. 1457–1505.

501 Arnqvist, G. *et al.* (2000) 'Sexual conflict promotes speciation in insects', *Proceedings of the National*
502 *Academy of Sciences*, 97(19), pp. 10460–10464. doi: 10.1073/pnas.97.19.10460.

503 Aron, S., Mardulyn, P. and Leniaud, L. (2016) 'Evolution of reproductive traits in *Cataglyphis* desert ants:
504 mating frequency, queen number, and thelytoky', *Behavioral ecology and sociobiology*. Springer, 70(8),
505 pp. 1367–1379. doi: 10.1007/s00265-016-2144-9.

506 Bolnick, D. I. and Fitzpatrick, B. M. (2007) 'Sympatric Speciation: Models and Empirical Evidence', *Annual*
507 *Review of Ecology, Evolution, and Systematics*, 38(1), pp. 459–487. doi:
508 10.1146/annurev.ecolsys.38.091206.095804.

509 Boulay, R. *et al.* (2017a) 'Social life in arid environments: the case study of *Cataglyphis* ants', *Annual*
510 *review of entomology*. Annual Reviews, 62, pp. 305–321.

511 Boulay, R. *et al.* (2017b) 'Social Life in Arid Environments: The Case Study of *Cataglyphis* Ants', *Annual*
512 *Review of Entomology*, 62(1), pp. 305–321. doi: 10.1146/annurev-ento-031616-034941.

513 Bourke, A. F. G. and Franks, N. R. (1995) *Social evolution in ants*. Princeton, New Jersey: Princeton
514 University Press.

515 Cognate, A. I., Seybold, S. J. and Sperling, F. A. H. (1999) 'Incomplete barriers to mitochondrial gene flow
516 between pheromone races of the North American pine engraver, *Ips pini* (Say)(Coleoptera, Scolytidae)',
517 *Proceedings of the Royal Society of London B: Biological Sciences*. The Royal Society, 266(1431), pp.
518 1843–1850.

519 Darras, H., Kuhn, A. and Aron, S. (2014) 'Genetic determination of female castes in a hybridogenetic

520 desert ant', *Journal of Evolutionary Biology*. Wiley Online Library, 27(10), pp. 2265–2271. doi:
521 10.1111/jeb.12470.

522 Earl, D. A. (2012) 'STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output
523 and implementing the Evanno method', *Conservation genetics resources*. Springer, 4(2), pp. 359–361.

524 Edgar, R. C. (2004) 'MUSCLE: multiple sequence alignment with high accuracy and high throughput',
525 *Nucleic acids research*. Oxford University Press, 32(5), pp. 1792–1797.

526 Eyer, P.-A. *et al.* (2013) 'Hybridogenesis through thelytokous parthenogenesis in two Cataglyphis desert
527 ants', *Molecular ecology*. Wiley Online Library, 22(4), pp. 947–955.

528 Eyer, P. A. *et al.* (2017) 'An integrative approach to untangling species delimitation in the Cataglyphis
529 bicolor desert ant complex in Israel', *Molecular phylogenetics and evolution*. Elsevier, 115, pp. 128–139.
530 doi: 10.1016/j.ympev.2017.07.024.

531 Eyer, P. A. and Hefetz, A. (2018) *Cytosuclear incongruences hamper species delimitation in the socially*
532 *polymorphic desert ants of the Cataglyphis albicans group in Israel*, *Journal of Evolutionary Biology*. doi:
533 10.1111/jeb.13378.

534 Feder, J. L., Egan, S. P. and Nosil, P. (2012) 'The genomics of speciation-with-gene-flow', *Trends in*
535 *Genetics*. Elsevier, 28(7), pp. 342–350. doi: 10.1016/j.tig.2012.03.009.

536 Fišer, C., Robinson, C. T. and Malard, F. (2018) 'Cryptic species as a window into the paradigm shift of
537 the species concept', *Molecular Ecology*, 27(3), pp. 613–635. doi: 10.1111/mec.14486.

538 Goudet, J. (1995) 'FSTAT (version 1.2): a computer program to calculate F-statistics', *Journal of heredity*.
539 Oxford University Press, 86(6), pp. 485–486.

540 Hefetz, A. (2007) 'The evolution of hydrocarbon pheromone parsimony in ants (Hymenoptera :

541 Formicidae) – interplay of colony odor uniformity and odor idiosyncrasy . A review', *Regulation*,
542 10(September), pp. 59–68. Available at:
543 http://myrmecologicalnews.org/cms/images/pdf/volume10/mn10_59-68_printable.pdf.

544 Hey, J. and Pinho, C. (2012) 'Population genetics and objectivity in species diagnosis', *Evolution*, 66(5),
545 pp. 1413–1429. doi: 10.1111/j.1558-5646.2011.01542.x.

546 Ionesco, A. and Eyer, P. A. (2016) 'Notes on Cataglyphis Foerster , 1850 of the bicolor species-group in
547 Israel , with description of a new species (Hymenoptera : Formicidae)', *Israel Journal of Entomology*,
548 46(December), pp. 109–131.

549 Jones, O. R. and Wang, J. (2010) 'COLONY: a program for parentage and sibship inference from
550 multilocus genotype data', *Molecular ecology resources*. Wiley Online Library, 10(3), pp. 551–555.

551 Jowers, M. J. *et al.* (2013) 'Social and Population Structure in the Ant Cataglyphis emmae', *PLoS ONE*,
552 8(9). doi: 10.1371/journal.pone.0072941.

553 Kather, R. and Martin, S. J. (2012) 'Cuticular hydrocarbon profiles as a taxonomic tool: Advantages,
554 limitations and technical aspects', *Physiological Entomology*, 37(1), pp. 25–32. doi: 10.1111/j.1365-
555 3032.2011.00826.x.

556 Kidokoro-Kobayashi, M. *et al.* (2012) 'Chemical Discrimination and Aggressiveness via Cuticular
557 Hydrocarbons in a Supercolony-Forming Ant, Formica yessensis', *PLoS ONE*, 7(10). doi:
558 10.1371/journal.pone.0046840.

559 Leniaud, L. *et al.* (2011) 'Multiple mating and supercoloniality in Cataglyphis desert ants', *Biological*
560 *Journal of the Linnean Society*, 104(4), pp. 866–876. doi: 10.1111/j.1095-8312.2011.01772.x.

561 Leniaud, L. *et al.* (2012) 'Social hybridogenesis in the clonal ant Cataglyphis hispanica', *Current biology*.

562 Elsevier, 22(13), pp. 1188–1193.

563 Lenoir, A. *et al.* (2009) ‘Cataglyphis desert ants: a good model for evolutionary biology in Darwin’s
564 anniversary year-A review’, *Israel Journal of Entomology*, 39, pp. 1–32. Available at:
565 <http://digital.csic.es/handle/10261/65135>.

566 Leppänen, J. *et al.* (2015) ‘Genetic divergence between the sympatric queen morphs of the ant *Myrmica*
567 *rubra*’, *Molecular Ecology*, 24(10). doi: 10.1111/mec.13170.

568 Matsubayashi, K. W., Ohshima, I. and Nosil, P. (2010) ‘Ecological speciation in phytophagous insects’,
569 *Entomologia experimentalis et applicata*. Wiley Online Library, 134(1), pp. 1–27.

570 Mayr, E. (2000) ‘The biological species concept’, *Species concepts and phylogenetic theory: a debate*.
571 *Columbia University Press, New York*, pp. 17–29.

572 Nosil, P. (2008) ‘Speciation with gene flow could be common’, *Molecular Ecology*. Wiley/Blackwell
573 (10.1111), 17(9), pp. 2103–2106. doi: 10.1111/j.1365-294X.2008.03715.x.

574 Nosil, P., Harmon, L. J. and Seehausen, O. (2009) ‘Ecological explanations for (incomplete) speciation’,
575 *Trends in ecology & evolution*. Elsevier, 24(3), pp. 145–156.

576 Pearcy, M. *et al.* (2004) ‘Characterization of nuclear DNA microsatellite markers in the ant *Cataglyphis*
577 *cursor*’, *Molecular Ecology Resources*. Wiley Online Library, 4(4), pp. 642–644.

578 Peeters, C. and Aron, S. (2017) ‘Evolutionary reduction of female dispersal in *Cataglyphis* desert ants’,
579 *Biological Journal of the Linnean Society*, 122(1), pp. 58–70. doi: 10.1093/biolinnean/blx052.

580 Pritchard, J. K., Stephens, M. and Donnelly, P. (2000) ‘Inference of population structure using multilocus
581 genotype data’, *Genetics*. Genetics Soc America, 155(2), pp. 945–959.

582 Purcell, J. *et al.* (2014) 'Convergent Genetic Architecture Underlies Social Organization in Ants', *Current*
583 *Biology*. Cell Press, 24(22), pp. 2728–2732. doi: <https://doi.org/10.1016/j.cub.2014.09.071>.

584 De Queiroz, K. (2007) 'Species concepts and species delimitation', *Systematic Biology*, 56(6), pp. 879–
585 886. doi: 10.1080/10635150701701083.

586 Radchenko, A. G. (2001) 'The phylogeny and faunogenesis of the genus *Cataglyphis* Foerster
587 (Hymenoptera, Formicidae)', *Entomological Review*. [Washington]: Published for the Entomological
588 Society of America by Scripta Pub. Co.[etc.], 1959-, 81(8), pp. 951–958.

589 Raymond, M. and Rousset, F. (1995) 'GENEPOP on the Web (Version 3.4)', URL: [http://wbiomed.curtin.](http://wbiomed.curtin.edu.au/genepop/Updated%20from%20Raymond%20&%20Rousset)
590 [edu. au/genepop/Updated from Raymond & Rousset](http://wbiomed.curtin.edu.au/genepop/Updated%20from%20Raymond%20&%20Rousset).

591 Reiner Brodetzki, T. *et al.* (2018) 'Demography and behaviour of polygyne nests of the supercolonial ant
592 *Cataglyphis niger*: Does kinship matter?', *bioRxiv*. Cold Spring Harbor Laboratory, p. 392522.

593 Ronque, M. U. V *et al.* (2016) 'Three ways to distinguish species: Using behavioural, ecological, and
594 molecular data to tell apart two closely related ants, *Camponotus renggeri* and *Camponotus rufipes*
595 (Hymenoptera: Formicidae)', *Zoological Journal of the Linnean Society*, 176(1), pp. 170–181. doi:
596 10.1111/zoj.12303.

597 Ross, K. G. (1997) 'Multilocus evolution in fire ants: effects of selection, gene flow and recombination',
598 *Genetics*. Genetics Soc America, 145(4), pp. 961–974.

599 Ross, K. G. *et al.* (2010) 'Species delimitation: A case study in a problematic ant taxon', *Systematic*
600 *Biology*, 59(2), pp. 162–184. doi: 10.1093/sysbio/syp089.

601 Ross, K. G. and Shoemaker, D. D. (1997) 'Nuclear and mitochondrial genetic structure in two social forms
602 of the fire ant *Solenopsis invicta*: Insights into transitions to an alternate social organization', *Heredity*,

603 78(6), pp. 590–602. doi: 10.1038/hdy.1997.98.

604 Ryan, S. F. *et al.* (2017) ‘Patterns of divergence across the geographic and genomic landscape of a
605 butterfly hybrid zone associated with a climatic gradient’, *Molecular ecology*. Wiley Online Library.

606 Saar, M. *et al.* (2014) ‘At the brink of supercoloniality: genetic, behavioral, and chemical assessments of
607 population structure of the desert ant *Cataglyphis niger*’, *Frontiers in Ecology and Evolution*, 2(May), pp.
608 1–10. doi: 10.3389/fevo.2014.00013.

609 Schwander, T., Keller, L. and Cahan, S. H. (2007) ‘Two alternate mechanisms contribute to the
610 persistence of interdependent lineages in *Pogonomyrmex* harvester ants’, *Molecular Ecology*, 16(17),
611 pp. 3533–3543. doi: 10.1111/j.1365-294X.2007.03407.x.

612 Seifert, B. (2009) ‘Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the
613 alpha taxonomic approach’, *Myrmecol. Nachr.*, 12(September), pp. 149–166. Available at:
614 http://www.myrmecologicalnews.org/cms/images/pdf/volume12/mn12_149-166_non-printable.pdf.

615 Seifert, B. (2010) ‘Intranidal mating, gyne polymorphism, polygyny, and supercoloniality as factors for
616 sympatric and parapatric speciation in ants’, *Ecological Entomology*, 35(SUPPL. 1), pp. 33–40. doi:
617 10.1111/j.1365-2311.2009.01136.x.

618 Shoemaker, D. D. and Ross, K. G. (1996) ‘Effects of social organization on gene flow in the fire ant
619 *Solenopsis invicta*’, *Nature*, pp. 613–616. doi: 10.1038/383613a0.

620 Simon, C. *et al.* (1994) ‘Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences
621 and a compilation of conserved polymerase chain reaction primers’, *Annals of the entomological Society
622 of America*. Oxford University Press Oxford, UK, 87(6), pp. 651–701.

623 Soroker, V. *et al.* (1994) ‘The postpharyngeal gland as a “Gestalt” organ for nestmate recognition in the

- 624 antCataglyphis niger', *Naturwissenschaften*, 81(11), pp. 510–513. doi: 10.1007/BF01132686.
- 625 Taylor, S. and Campagna, L. (2016) 'Avian supergenes', *Science*, 351(6272), p. 446 LP-447. Available at:
- 626 <http://science.sciencemag.org/content/351/6272/446.abstract>.
- 627 Timmermans, I. *et al.* (2010) 'Mating system and population structure in the desert ant Cataglyphis
- 628 livida', *Insectes Sociaux*, 57(1), pp. 39–46. doi: 10.1007/s00040-009-0048-7.
- 629 Torres, C. W. *et al.* (2018) 'Genetic and chemical divergence among host races of a socially parasitic ant',
- 630 *Ecology and Evolution*. John Wiley & Sons, Ltd, 0(0). doi: 10.1002/ece3.4547.
- 631 Wang, J. (2011) 'COANCESTRY: a program for simulating, estimating and analysing relatedness and
- 632 inbreeding coefficients', *Molecular ecology resources*. Blackwell Publishing Ltd, 11(1), pp. 141–145.
- 633 Wang, J. *et al.* (2013) 'A Y-like social chromosome causes alternative colony organization in fire ants',
- 634 *Nature*. Nature Publishing Group, 493(7434), pp. 664–668. doi: 10.1038/nature11832.
- 635 Wondji, C., Simard, F. and Fontenille, D. (2002) 'Evidence for genetic differentiation between the
- 636 molecular forms M and S within the Forest chromosomal form of Anopheles gambiae in an area of
- 637 sympatry', *Insect molecular biology*. Wiley Online Library, 11(1), pp. 11–19.

638

639 **Figure and Tables with captions**

Figure 1: STRUCTURE results of the ddRADseq, possible K outcomes 5-7. *C. israelensis* in light blue, the *C. niger* complex: *C. drusus*, *C. niger*, and *C. savignyi* in orange, *C. holgerseni* in green or pink, *C. isis* in dark blue, and *C. noda* in green.

Figure 2: A) STRUCTURE harvester results for the Betzet population indicating the best probable K is K=2, then K= 3 and K=13. B) The population STRUCTURE divisions according to the most probable K possibilities. C) Principle Components Analysis of genotypes colored according to STRUCTURE result k=3 D) Locations of estimated colonies based on similar assumed matriline according to COLONY; same color indicates the same matriline, light blue circle with black edge indicates a colony with no other similar matriline. E) Discriminant Analysis of CHC groups are set according to k=3. F) K- means of CHC profiles K=4, individuals are marked with nest number (first 3 digits) and individual ID. Circle indicates k-1, triangle k-2, cross k-3, and X k-4. The two components explain 70.93% of the variation.

Figure 3: 22 nests that were collected for the behavioral essays. Each circle indicates a physical nest, while each color indicates a different colony obtained by the merging assays. Queen-right nests are

656 indicated by a large circle. Assays were performed between all colonies as indicated in Table 2. Nests
657 that merged are indicated by a green line; nests that did not merge are indicated by a red line.

659 Figure 4: A) STRUCTURE harvester results for the Tel Baruch population indicating the best probable K is
 660 K=2, then K= 3 and K=6. B) The population STRUCTURE divisions according to the most probable K
 661 possibilities. C) STRUCTURE results for the Tel Baruch population of K=3 plotted on the map, the
 662 compression with the mitotypes plotted in (D) exemplifies that the STRUCTURE division is not related to
 663 the mitochondrial DNA. D) Plotted on the map of the Tel Baruch sampling site Cytochrome B
 664 phylogenetic clades. E) Principle Components Analysis of genotypes for Tel Baruch population colored
 665 according to the different mitotypes. F) Discriminant analysis results of CHC's composition for the Tel
 666 Baruch population according to the three mitotypes.

668 Figure 5: Location of nests used for the merging experiment. Nests are colored according to the
669 mitotype: blue- *niger*, green- *savignyi*, and yellow- *drusus*. The lines drawn between nests indicate if the
670 nests merged (green) or not (red).

Figure 6: Bayesian inference tree CytB haplotypes of sperm content from *niger* queens. Bayesian probabilities/bootstrap values (from Ugene) are given to estimate branch support. The trees are rooted using the haplotype sequences from Eyer *et al.* (2017).

Supplementary figures:

Figure S1: comparison of reads coverage of each ant at the stage of raw reads (red) and after the alignment based filtering (blue). Notice that the initial coverage is based on averaging the number of reads over the known number of restriction sites, while the final coverage is based on actual mapping of the reads to the reference genome of *C. drusus* and is therefore more accurate.

Figure S2: A) Typical cuticular hydrocarbon profile of the *drusus* mitotype from Betzet. The chromatogram shows only the 60 identified compounds (numbered peaks), of which 35 hydrocarbons were used in the analysis (marked in bold). B) The cuticular hydrocarbon profile from the Tel Baruch population CHC's used for the analysis is marked in bold (44 out of 74 identified).

691 Tables:

692 Table 1: Gene flow 3S test. 3S run results comparing two coalescent models for different triplets of
 693 species. The difference shown is between the highest scoring alternative model and the highest scoring
 694 null model between 3 runs for each of the species combination. The bold lines are for species triplets,
 695 for which the null model was rejected, suggesting gene flow between the first two species.

Species combination	loci Nr.	2DlnL	pvalue
<i>C. israelensis</i> : <i>C. savignyi</i> , <i>C. isis</i>	23,031	0	>0.99
<i>C. israelensis</i> : <i>C. niger</i> , <i>C. isis</i>	22,770	4.933	0.1
<i>C. israelensis</i> : <i>C. drusus</i> , <i>C. isis</i>	23,662	0.55	0.975
<i>C. savignyi</i> : <i>C. niger</i> , <i>C. israelensis</i>	27,769	103.99	<0.001
<i>C. savignyi</i> : <i>C. drusus</i> , <i>C. israelensis</i>	28,830	384.18	<0.001
<i>C. niger</i> : <i>C. drusus</i> , <i>C. israelensis</i>	28,626	57.399	<0.001
<i>C. Holgerseni</i> : <i>C. savignyi</i> , <i>C. isis</i>	22,771	21.17	<0.001
<i>C. savignyi</i> : <i>C. isis</i> , <i>C. holgerseni</i>	23,561	0.008	>0.99

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698 Table 2: Merging assays of the Betzet population

Nest A	Nest B	Merging	Between-colony trophallaxis	Between-colony aggression	Maximal distance between colonies (meters)
1	9	+	+	-	1.5
2	3	+	+	-	2
4	5	+	+	-	1.5
14	15	+	+	+	2
10	11	+	+	+	2.3
16	17	+	+	+	10.4
6	4+5	+	+	+	4.5
8	13	-	-	+	14
12	13	-	-	+	20
8	12	+	+	-	18
2+3	4+5+6	-	-	+	34
1+9	4+5+6	+	+	-	8.5
1+9	2+3	-	-	+	10
16+17	14+15	-	-	+	37
10+11	2+3	-	-	+	36
16+17	18	+	+	-	9.4
14+15	12+8	-	-	+	15-50
1+9+4+5+6	10+11	-	-	+	20
1+9+4+5+6	12+8	-	-	+	37
1+9+4+5+6	7	+	+	-	22
10+11	12+8	+	+	-	55
14+15	13	+	+	-	42
14+15	10+11	-	-	+	75-112

10+11	16+17+18	+	+	-	140
2+3	7	-	-	+	34
7 (QR)	10+11 (QR)	-	-	+	43
7 (QR)	16+17+18 (QR)				90

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Table 3: Microsatellites analysis of the Tel Baruch population when divided according to the different mitotypes.

Haplotype	Fit	Fit in a single mitotype population	Fst	Fst in a single mitotype population	Fis	Within nest Relatedness	Within nest Relatedness in a single mitotype population
All nests/haplotypes n=26	0.022±0.013		0.117±0.008		0.108±0.008	0.229±0.013	
niger n=6	0.051±0.02	0.023±0.008 ¹	0.059±0.005	0.005±0.003 ¹	0.008±0.019	0.112±0.009	0.02±0.007 ¹
drusus n=3	0.027±0.033	0.09±0.06 ²	0.092±0.008	0.19±0.015 ²	0.131±0.029	0.19±0.013	0.35 ±0.02 ²
savignyi n=17	0.03±0.011	0.031 ³	0.143±0.013	0.151 ³	0.132±0.015	0.278±0.023	0.247 ³

¹Population is located in Nitzanim (Leniaud *et al.*, 2011)

² Population is located in Betzet (This paper)

³ Population is located in Arad, Rishon and Palmachim (Leniaud *et al.*, 2011; Saar *et al.*, 2014)

Table 4: Merging assays of the Tel Baruch population

Nest A (mitochondrial haplotype)	Nest B (mitochondrial haplotype)	Merging	Between- colony trophallaxis	Between- colony aggression	Maximal distance between colonies (meters)
1 (<i>Niger</i>)	2 (<i>Niger</i>)	+	+	-	100
3 (<i>Niger</i>)	4 (<i>Niger</i>)	+	+	-	100
1+2 (<i>Niger</i>)	3+4 (<i>Niger</i>)	+	+	-	400
5 (<i>Niger</i>)	7 (<i>Drusus</i>)	-	-	+	650
6 (<i>Niger</i>)	8 (<i>Savignyi</i>)	-	-	+	450
7 (<i>Drusus</i>)	8 (<i>Savignyi</i>)	-	-	+	35
9 (<i>Savignyi</i>)	10 (<i>Savignyi</i>)	-	-	+	35

Supplementary Tables:

Table 3.S1: GPS coordinates of samples used in the study.

Betzet population		Tel Baruch population		
525	N33 04.651 E35 06.539	454	N32 07.637 E34 47.269	
526	N33 04.651 E35 06.537	455	N32 07.624 E34 47.288	
527	N33 04.650 E35 06.536	457	N32 07.606 E34 47.270	
528	N33 04.675 E35 06.582	461	N32 07.634 E34 47.242	
530	N33 04.601 E35 06.534	462	N32 07.592 E34 47.192	
531	N33 04.615 E35 06.522	464	N32 07.613 E34 47.208	
532	N33 04.619 E35 06.526	465	N32 07.601 E34 47.213	
533	N33 04.586 E35 06.507	466	N32 07.604 E34 47.217	
534	N33 04.612 E35 06.545	467	N32 07.595 E34 47.211	
535	N33 04.600 E35 06.540	469	N32 07.572 E34 47.248	
538	N33 04.584 E35 06.541	473	N32 07.577 E34 47.254	
539	N33 04.635 E35 06.510	481	N32 07.592 E34 47.204	
540	N33 04.658 E35 06.502	487	N32 07.628 E34 47.215	
541	N33 04.561 E35 06.507	489	N32 07.620 E34 47.198	
542	N33 04.563 E35 06.509	491	N32 07.601 E34 47.186	
543	N33 04.559 E35 06.521	498	N32 07.578 E34 47.188	
544	N33 04.579 E35 06.536	499	N32 07.561 E34 47.154	
545	N33 04.582 E35 06.525	500	N32 07.562 E34 47.151	
546	N33 04.585 E35 06.526	505	N32 07.573 E34 47.164	
547	N33 04.585 E35 06.526	506	N32 07.572 E34 47.162	
548	N33 04.589 E35 06.523	599	N32 07.616 E34 47.254	
549	N33 04.567 E35 06.495	600	N32 07.582 E34 47.258	
550	N33 04.572 E35 06.513	602	N32 07.559 E34 47.161	
551	N33 04.585 E35 06.513	603	N32 07.557 E34 47.238	
552	N33 04.665 E35 06.483	604	N32 07.558 E34 47.235	
553	N33 04.674 E35 06.560	606	N32 07.554 E34 47.243	
554	N33 04.672 E35 06.562	608	N32 07.616 E34 47.186	
555	N33 04.678 E35 06.449	640	N32 16.934 E34 50.419	
556	N33 04.678 E35 06.449	641	N32 07.454 E34 47.125	
557	N33 04.679 E35 06.448	642	N32 07.455 E34 47.116	
559	N33 04.671 E35 06.450	643	N32 07.512 E34 47.146	
560	N33 04.627 E35 06.455	644	N32 07.496 E34 47.162	
562	N33 04.628 E35 06.460	645	N32 07.537 E34 47.124	
563	N33 04.652 E35 06.543	648	N32 07.682 E34 47.294	
564	N33 04.635 E35 06.554	650	N32 07.681 E34 47.281	
565	N33 04.634 E35 06.557	653	N32 07.951 E34 47.432	
568	N33 04.586 E35 06.485	654	N32 07.933 E34 47.420	
570	N33 04.654 E35 06.533	655	N32 07.944 E34 47.416	
571	N33 04.653 E35 06.534	657	N32 07.770 E34 47.309	
575	N33 04.595 E35 06.404	658	N32 07.775 E34 47.292	
576	N33 04.604 E35 06.405	Merging samples-Betzet		
577	N33 04.601 E35 06.405	2	969	N33 04.598 E35 06.566
578	N33 04.511 E35 06.401	3	970	N33 04.598 E35 06.567
579	N33 04.507 E35 06.406	1	971	N33 04.608 E35 06.554
580	N33 04.445 E35 06.379	4	973	N33 04.609 E35 06.549
581	N33 04.440 E35 06.412	5	974	N33 04.610 E35 06.549
582	N33 04.366 E35 06.377	7	976	N33 04.600 E35 06.542
583	N33 04.370 E35 06.392	6	977	N33 04.607 E35 06.549
584	N33 04.373 E35 06.392	8	979	N33 04.585 E35 06.545
585	N33 04.323 E35 06.370	9	981	N33 04.607 E35 06.554
586	N33 04.328 E35 06.375	10	982	N33 04.618 E35 06.561
587	N33 04.269 E35 06.372	11	983	N33 04.618 E35 06.560
588	N33 04.267 E35 06.379	13	985	N33 04.583 E35 06.537
589	N33 04.175 E35 06.358	12	986	N33 04.594 E35 06.540
590	N33 04.196 E35 06.352	16	987	N33 04.566 E35 06.499
595	N33 04.097 E35 06.315	15	988	N33 04.564 E35 06.524
596	N33 04.088 E35 06.318	14	989	N33 04.563 E35 06.524
		17	990	N33 04.565 E35 06.493
		18	991	N33 04.564 E35 06.501

Sample name	Mitotype	Locaion	North	East	Collected by
11	Drusus	Betzet	N334.633	E356.509	TRB
21	Drusus	Bostan hagaili	N3257.208	E354.514	TRB
31	Drusus	Kiryat haim	N3257.277	E354.529	TRB
46	Israelensis	Hof hacarmel	N3246.951	E3457.358	TRB
51	Savignyi	Zikim	N3136.807	E3431.365	TRB
61	Niger	Zikim west	N3136.812	E3431.303	TRB
76	Savignyi	Askelon	N3140.471	E3433.256	TRB
86	complex	Nitzan	N3144.647	E3437.954	TRB
100	complex	Nitzan	N3144.673	E3437.92	TRB
116	Savignyi	Palmachim	N3155.92	E3443.322	TRB
146	Savignyi	Rishon	N3158.874	E3444.517	TRB
211	Israelensis	Tyrat carmel	N3246.791	E3457.334	TRB
241	Savignyi	Ceseria	N3230.84	E3453.892	TRB
454	Niger	Tel Baruch	N327.637	E3447.269	TRB
461	Niger	Tel Baruch	N327.634	E3447.242	TRB
464	savignyi	Tel Baruch	N327.613	E3447.208	TRB
465	complex	Tel Baruch	N327.601	E3447.213	TRB
469	Savignyi	Tel Baruch	N327.572	E3447.248	TRB
481	savignyi	Tel Baruch	N327.592	E3447.204	TRB
486	niger	Tel Baruch	N327.629	E3447.216	TRB
487	niger	Tel Baruch	N327.628	E3447.215	TRB
489	savignyi	Tel Baruch	N327.62	E3447.198	TRB
491	niger	Tel Baruch	N327.601	E3447.186	TRB
499	savignyi	Tel Baruch	N327.561	E3447.154	TRB
500	Savignyi	Tel Baruch	N327.562	E3447.151	TRB
505	Savignyi	Tel Baruch	N327.573	E3447.164	TRB
600	savignyi	Tel Baruch	N327.582	E3447.258	TRB
602	savignyi	Tel Baruch	N327.559	E3447.161	TRB
608	Niger	Tel Baruch	N327.616	E3447.186	TRB
641	Drusus	Tel Baruch	N327.454	E3447.125	TRB
642	Drusus	Tel Baruch	N327.455	E3447.116	TRB
643	Savignyi	Tel Baruch	N327.512	E3447.146	TRB
644	Drusus	Tel Baruch	N327.496	E3447.162	TRB
650	Niger	Tel Baruch	N327.681	E3447.281	TRB
653	Niger	Tel Baruch	N327.951	E3447.432	TRB
657	complex	Tel Baruch	N327.77	E3447.309	TRB
922	Israelensis	Atlit	N3242.587	E3456.623	TRB
993	Israelensis	Tveria	N3248.044	E3532.211	TRB
994	Israelensis	Tveria	N3248.021	E3532.223	TRB
995	Israelensis	Amiad	N3254.951	E3532.76	TRB
996	Israelensis	Amiad	N3254.952	E3532.761	TRB
997	Israelensis	Agmon	N336.708	E3535.079	TRB
999	Savignyi	Vingate	N3215.381	E3449.7	TRB
1000	Savignyi	Vingate	N3215.381	E3449.7	TRB
1001	Savignyi	Vingate	N3215.381	E3449.7	TRB
498	Savignyi	Tel Baruch	N327.578	E3447.188	TRB
506	Savignyi	Tel Baruch	N327.572	E3447.162	TRB
641tb	Drusus	Tel Baruch	N327.454	E3447.125	TRB
642tb	Drusus	Tel Baruch	N327.455	E3447.116	TRB
85Shab	Israelensis	Habonim	N3238.932	E3455.664	TRB
anm1	Israelensis	Allonim	32°42'40.2"N	35°08'33.9"E	Eyer et al. 2017
ash101	Savignyi	Ashdod	N3149.883	E3439.777	Eyer et al. 2017
asv1	Israelensis	Azmon Segev	32°51'47.6"N	35°15'13.1"E	Eyer et al. 2017
bim1	Israelensis	Bir El Maksur	32°46'05.5"N	35°14'08.2"E	Eyer et al. 2017
bmh3	Isis	Be'er Menuha	30°18'3.71"N	35°7'53.97"E	Eyer et al. 2017
dha1	Israelensis	Deir Hanna	32°51'35.7"N	35°21'35.7"E	Eyer et al. 2017
dov3	Israelensis	Daverat	32°39'14.2"N	35°22'39.7"E	Eyer et al. 2017
eha1	Israelensis	En Harod (Me'uhad)	32°33'21.6"N	35°22'59.3"E	Eyer et al. 2017
ehn4	Israelensis	En HaNaziv	32°28'36.4"N	35°29'54.6"E	Eyer et al. 2017
ely3	Israelensis	Elyagim	32°37'51.2"N	35°04'10.4"E	Eyer et al. 2017
evd	Isis	En 'Avedat	30°50'49.99"N	34°46'45.46"E	Eyer et al. 2017
eya1	Holgerseni	En Yahav 2	30°39'51.32"N	35°15'36.72"E	Eyer et al. 2017
eyh1	Holgerseni	En Yahav	30°39'44.79"N	35°14'19.59"E	Eyer et al. 2017
gaa3	Savignyi	Ga'ash	32°14'25.50"N	34°49'44.63"E	Eyer et al. 2017
ghs	Savignyi	Gan hashomron	32°28'04.3"N	34°59'25.2"E	Eyer et al. 2017
gid1	Israelensis	Gid'ona	32°32'48.3"N	35°21'30.2"E	Eyer et al. 2017
hbk1	Israelensis	Ha---Biq'a	32°03'01.7"N	35°27'44.2"E	Eyer et al. 2017
her1	Savignyi	Hertzelia	32°09'45.36"N	34°48'03.07"E	TRB
her2	Savignyi	Hertzelia	32°09'45.36"N	34°48'03.07"E	TRB
hoa3	Israelensis	Horbat 'Ammudim	32°48'46.4"N	35°24'25.3"E	Eyer et al. 2017
hzy1	Israelensis	HaZayit	32°36'44.8"N	35°00'20.6"E	Eyer et al. 2017
hzy1_2	Niger	Jaffa	32°36'44.8"N	35°00'20.6"E	Eyer et al. 2017
jaf1	Niger	Jaffa	32°24'35.91"N	34°44'48.63"E	Eyer et al. 2017
jaf2	Niger	Jaffa	32°24'35.91"N	34°44'48.63"E	Eyer et al. 2017
jer1	Israelensis	Jerusalem	31°45'8.94"N	35°12'50.50"E	Eyer et al. 2021
maon	unknown	Ma'aon	31°24'52.53"N	35°09'50.01"E	Friedman L.
mae1	Israelensis	Ma'ale Efrayim	32°03'58.6"N	35°23'59.0"E	Eyer et al. 2017
mhe	Israelensis	Mishmar Ha'Emeq	32°36'47.1"N	35°08'50.3"E	Eyer et al. 2017
mic1	Savignyi	Michmoret	32°23'51.50"N	34°52'13.60"E	TRB
mic2	Savignyi	Michmoret	32°23'51.50"N	34°52'13.60"E	TRB
mzr1	unknown	Mizpe Ramon	30°36'18.11"N	34°51'57.24"E	Rozenberg T.
mzr3_2	unknown	Mizpe Ramon	30°36'18.11"N	34°51'57.24"E	Rozenberg T.
mzr4	unknown	Mizpe Ramon	30°36'18.11"N	34°51'57.24"E	Rozenberg T.
NEG2	Isis	Negev point 880	30°18'0.08"N	34°58'6.16"E	Eyer et al. 2017
NET3	Savignyi	Netanya	32°18'40.2"N	34°50'43.2"E	Eyer et al. 2017
ngv3	unknown	Negev point 881	30°26'25.76"N	34°56'19.55"E	Eyer et al. 2017
pol633	savignyi	Poleg	N3247.218	E351.229	TRB
pol634	savignyi	Poleg	N3216.877	E3450.31	TRB
pol635	savignyi	Poleg	N3216.906	E3450.333	TRB
pol636	savignyi	Poleg	N3216.897	E3450.334	TRB
pol637	savignyi	Poleg	N3216.915	E3450.417	TRB
poleg638	savignyi	Poleg	N3216.933	E3450.42	TRB
qed	Israelensis	Qedumim	32.212896	35.159068	Friedman L.
revpa6	Savignyi	Revivim	31°02'39.2"N	34°43'34.0"E	Eyer PA
sat	Savignyi	Sattaf	31°46'18.6"N	35°07'38.9"E	Eyer et al. 2017
sdo1	Savignyi	Sedom	31°03'27.26"N	35°23'02.13"E	Renan I.
sus	Savignyi	Susya	31°23'39.07"N	35°07'01.00"E	Friedman L.
taf	Savignyi	tel Afek	32°7'32.43"N	34°53'44.21"E	Eyer et al. 2017
tam2	Israelensis	Tamra	32°52'31.2"N	35°10'33.7"E	Eyer et al. 2017

Table S2: The number of nests used in each of the analysis.

Location	QL nests	QR nests	Nests in merging experiment	Nests in microsat analysis	Nests in CHC analysis
Betzet	76	5	22	57	49
Tel Baruch	39	21	10	26	28

Table S3: Assumed matriline according to COLONY in the Betzet population.

Nest #	Cn02		Cn02		Ch08		Cc54		Ch12		Ch01		Ch11		Ch10	
543	90	90	105	113	130	136	213	213	93	97	176	194	235	235	274	304
550	90	90	105	113	130	136	213	213	93	97	176	194	235	247	274	304
579	90	90	109	113	136	136	207	217	117	117	174	180	233	235	304	310
578	90	90	113	113	136	136	207	217	117	117	174	178	233	235	304	310
533	90	90	111	111	128	130	209	219	85	103	176	176	235	249	304	310
551	90	90	111	111	128	130	209	219	85	103	176	176	235	249	304	310
565	90	90	111	111	128	130	207	213	85	85	176	180	245	249	298	298
564	90	90	111	111	128	130	207	213	85	85	176	180	245	249	298	298
525	90	90	111	113	130	132	213	219	0	0	174	178	233	235	286	298
527	90	90	111	113	130	132	213	219	0	0	174	178	233	235	286	298
570	90	90	111	113	130	132	213	219	0	0	174	174	233	235	286	298
577	90	90	111	113	128	136	209	217	93	117	174	176	233	235	262	298
576	90	90	111	113	128	136	209	217	93	117	174	176	233	235	262	298
535	90	90	109	111	134	136	211	219	93	117	174	176	247	249	262	306
530	90	90	109	111	134	136	211	219	93	117	174	176	247	249	262	306
560	90	90	111	119	128	136	209	217	85	85	176	178	233	245	286	304
562	90	90	111	119	128	136	209	209	85	85	176	178	233	245	286	304
544	90	107	113	119	128	130	209	213	0	0	174	178	247	247	286	304
538	90	107	113	119	128	130	209	213	0	0	174	178	247	247	286	304
545	90	107	111	111	130	136	213	219	85	103	176	178	247	251	274	314
546	90	107	111	111	130	136	213	219	0	0	176	178	247	251	274	314
547	90	107	111	111	130	136	213	219	0	0	176	178	247	251	274	314
548	90	107	111	111	130	136	213	219	0	0	176	178	247	251	274	314
581	90	90	105	113	130	130	209	211	93	103	174	176	235	245	286	298
589	90	90	105	113	128	136	213	217	85	117	174	219	233	233	286	298
563	90	90	111	111	130	132	213	219	0	0	174	178	233	247	286	298
532	90	90	111	113	136	136	209	217	0	0	174	174	233	249	286	298
554	90	90	113	119	130	136	209	217	0	0	174	178	235	235	298	298
531	90	90	105	113	128	136	209	217	93	97	174	176	233	249	286	298
549	90	90	111	119	128	136	207	217	0	0	174	176	249	249	286	298
542	90	90	105	113	130	136	209	213	93	97	176	194	235	249	274	304
585	90	100	111	113	130	134	207	219	93	97	176	180	233	235	298	314
588	90	107	111	119	128	128	209	213	0	0	174	174	233	247	298	314
586	90	90	113	119	130	136	211	213	87	117	176	176	233	247	274	298
580	90	90	109	113	130	134	207	209	85	85	174	196	235	235	274	314
568	90	90	111	111	134	136	211	215	85	97	174	176	233	245	274	298
571	90	90	111	113	132	136	213	219	85	93	174	176	233	235	286	298
526	90	90	111	113	130	132	213	219	85	85	174	190	233	235	286	298
559	90	107	109	113	130	130	209	209	0	0	174	178	235	245	298	304
557	90	90	111	119	130	136	209	217	85	83	174	178	233	247	298	304
555	90	90	111	119	130	136	209	217	0	0	174	178	233	249	298	304
556	90	90	111	119	130	136	209	217	0	0	174	176	233	249	298	304
528	88	90	105	109	128	134	207	211	87	117	174	176	247	251	298	298
575	90	90	111	113	128	136	209	217	0	0	174	176	233	235	262	298
553	90	90	113	119	130	136	209	209	0	0	180	196	235	247	298	298
587	90	90	113	119	134	136	209	209	0	0	174	174	233	247	286	304
582	90	90	105	109	128	136	217	217	0	0	176	176	233	233	307	307
583	90	90	105	109	128	136	211	217	0	0	176	196	233	253	262	298
541	90	90	105	113	130	136	213	213	0	0	176	194	235	235	274	304
534	90	90	105	113	134	136	211	217	0	0	174	196	247	247	274	307
539	90	90	105	111	130	134	209	213	0	0	176	176	245	247	274	298
590	90	90	109	111	128	136	209	209	0	0	174	176	235	245	274	286
596	90	90	109	111	130	130	207	209	0	0	174	174	233	235	298	298
552	90	90	111	111	134	134	209	217	0	0	174	176	233	247	298	298
584	90	90	105	105	128	136	211	217	0	0	176	196	233	253	262	298
595	90	107	111	113	128	136	209	211	0	0	176	176	233	249	292	314
540	90	107	105	109	130	136	217	219	0	0	174	176	233	235	298	298