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Large-scale distribution of hybridogenetic lineages in a Spanish desert ant

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Recently, a unique case of hybridogenesis at a social level was reported in local populations of the desert ants *Cataglyphis*. Queens mate with males originating from a different genetic lineage than their own to produce hybrid workers, but they use parthenogenesis for the production of reproductive offspring (males and females). As a result, non-reproductive workers are all inter-lineage hybrids, whereas the sexual line is purely maternal. Here, we show that this unorthodox reproductive system occurs in all populations of the ant *Cataglyphis hispanica*. Remarkably, workers are hybrids of the same two genetic lineages along a 400 km transect crossing the whole distribution range of the species. These results indicate that social hybridogenesis in *C. hispanica* allows their maintenance over time and across a large geographical scale of two highly divergent genetic lineages, despite their constant hybridization. The widespread distribution of social hybridogenesis in *C. hispanica* supports that this reproductive strategy has been evolutionarily conserved over a long period.

1. Introduction

Hybridogenesis, or hemi-clonality, is an unusual mode of reproduction whereby a hybrid female expresses the paternal genome that she inherited from her father, yet she fails to transmit that paternal genome to her offspring [1,2]. During oogenesis, the chromosome sets of the parents fail to recombine and the paternal genome is excluded from the ova. As a consequence, both the maternal and paternal genomes are expressed in somatic tissues, whereas only the maternal genome constitutes the germ line and is perpetuated across generations. Hybridogenesis has been reported in several taxa of vertebrates, including *Poeciliopsis* teleost fishes [2], *Rana* waterfrogs [3] and *Bacillus* stick-insects [4], where it has been shown to stem from hybridization between different species.

Remarkably, a form of hybridogenesis at the social level has been reported in some ant species [5–13]. The hallmark of ant societies is reproductive division of labour based on a system of caste differentiation. Workers usually forgo their own direct reproduction and perform all the tasks required for colony development and growth (foraging, defending and caring for the brood), whereas queens and males are responsible for reproducing the colony [14]. In hybridogenetic ants, populations consist of self-sustainable pairs of perpetually hybridizing genetic lineages. Queens of each lineage mate with males originating from the co-occurring alternative lineage to produce hybrid workers. By contrast, reproductive individuals arise from intra-lineage mating or parthenogenesis and maintain pure-lineage genomes. Such a reproductive system results in a strong caste–genotype association (see reviews [15,16]).

Various inheritance modes have been described in ants with interdependent lineages. In the *Pogonomyrmex barbatus*/*Pogonomyrmex rugosus* species complex [10–12] and in *Solenopsis xyloni* × *Solenopsis geminata* [13], both female castes are produced by sexual reproduction: inter-lineage mating produces workers, whereas intra-lineage mating produces queens. Therefore, colonies must contain both inter- and intra-lineage mating pairs to produce the two female castes. Colonies of *Pogonomyrmex* are headed by single queens mated multiple times with males from their own as well as the alternative lineage. By contrast, colonies of *S. xyloni* contain multiple queens, each mated with a single male. Queens mated with a male of their own species produce only reproductive females (new

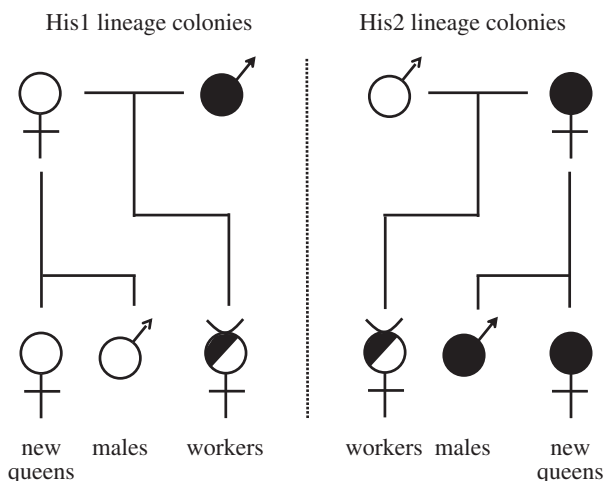


Figure 1. Social hybridogenesis in *C. hispanica*. Two genetic lineages, His1 and His2, coexist in each population of *C. hispanica*. Pure-lineage queens mate with a male originating from the alternative lineage than their own and use its sperm to produce inter-lineage sterile workers. By contrast, queens only use parthenogenesis for colony reproduction (i.e. new queens and males production). As a consequence, reproductive lines maintain non-recombinant pure-lineage genomes over time. Black and white colours represent nuclear genomes from the two genetic lineages.

queens), whereas queens mated with an *S. geminata* male produce only workers. Because males of Hymenoptera arise from unfertilized haploid eggs by arrhenotokous parthenogenesis, they belong to the queen lineage. Another type of dependent-lineage system has been reported in *Vollenhovia emeryi* [6], *Paratrechina longicornis* [5] and invasive populations of *Wasmania auropunctata* [7]. In these species, reproductives develop from eggs with a single genome: new queens are clones of their mothers, whereas males are clones of their fathers. The queen and male genomes are therefore completely isolated, whereas workers combine alleles from both maternal and paternal clonal lineages.

Recently, social hybridogenesis was discovered in some populations of *Cataglyphis* desert ants (figure 1). In *Cataglyphis hispanica* [9], *Cataglyphis mauritanica* and *Cataglyphis velox* [8], queens mate with one or a few males originating from an alternative lineage than their own. Workers are all inter-lineage hybrids, whereas both male and female reproductives are produced by the colony queen(s) through parthenogenesis. Thus, in contrast with the species above, males never transmit their genes to reproductive offspring, rather to workers only (figure 1). Social hybridogenesis in *Cataglyphis* ants is analogous to ‘classical’ hybridogenesis, because both the maternal and paternal genomes are expressed in a non-reproductive worker force (which corresponds to the soma of solitary organisms), while the sexual offspring (which correspond to the germ line of solitary organisms) are purely maternal.

Despite the frequent occurrence of social hybridogenesis in ants, the distribution and characterization of genetic lineages within species remains poorly studied. *Pogonomyrmex* harvester ants are currently the best-understood models of hybridogenesis. This hybrid system occurs in populations overlapping the range of the parental species *P. barbatus* and *P. rugosus* and four pairs of reproductively isolated dependent lineages have been identified [17,18]. Here, we studied the phylogeographic distribution of hybridogenetic lineages in the desert ant *C. hispanica*. Social hybridogenesis in this species has only been described in four nearby populations that are

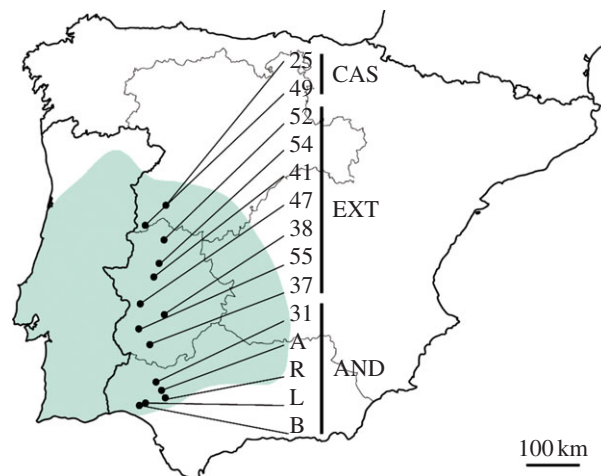


Figure 2. Sampled sites in southwest Spain; data for each site are given in table 1. The complete range of *C. hispanica* is shown in green [19]. Geographical zones are abbreviated as follows: Andalusia (AND), Extremadura (EXT) and Castile & León (CAS).

characterized by the same two genetic lineages. Whether this reproductive system occurs in other populations and whether additional lineages exist were unknown. We conducted a genetic survey of colony structure across the species’ distribution range. We also determined the number of queens per colony, queen-mating frequency, mode (sexual or asexual) of worker and queen production. Our genetic analyses indicated that all populations show the signature of social hybridogenesis: workers are all inter-lineage hybrids arising from sexual reproduction, whereas male and female sexuals are produced by parthenogenesis. Remarkably, *C. hispanica* consists of only two interdependent genetic lineages across its whole distribution range. This indicates that hybridogenesis in *C. hispanica* allows the maintenance of two divergent genetic lineages over time and across a large geographical scale, despite constant hybridization. To the best of our knowledge, this is the first description of a hybridogenetic reproductive system distributed across the entire range of a species.

2. Material and methods

(a) Biological model and sampling

Cataglyphis hispanica is found in the most arid habitats of the southwest of the Iberian Peninsula. In previously studied Andalusian populations, colonies were found to be headed by a single queen mated once [9]. Dispersal of colonies proceeds by budding: young mated queens leave the natal nest with the help of worker sisters and establish new colonies nearby.

To study the phylogeographic distribution of hybridogenetic lineages, we collected 68 colonies from 14 populations of *C. hispanica* along a 400 km north–south transect across the entire range of the species (figure 2). The number of colonies sampled per site ranged from three to seven (table 1). Colonies were excavated at the time of sexual production, in May 2012. Alate (virgin) queens and a sample of workers from each nest were directly preserved in 95% ethanol for subsequent genetic analyses. Mated wingless queens were taken alive to the laboratory for fresh dissection of their spermathecae.

(b) DNA isolation

DNA was extracted from adult ants by the Chelex 100 method [9]. To determine the number of mates of each queen and their

Table 1. Collection sites, sampling information and colony kin structure in *C. hispanica*. (The number of colonies sampled, the number of genotypes analysed for the mother queens and their mates (males), as well as the mean number of queens per colony and mating frequency are indicated for each collection site. Because foundation of new colonies proceeds by budding, workers in newly started colonies are not daughters of the resident queen but derive from the queen of the mother nest. In this situation, the number of genotypes inferred from workers is indicated in brackets.)

site	locality	colonies	queens (incl. inferred)	males (incl. inferred)	queen/colony (range)	males/queen (range)
B ^a	Bonares	5	5	5	1	1
L ^a	Lucena del Puerto	5	5	5	1	1
A ^a	Aznalcóllar	5	5	7 (2)	1	1.25 (1–2)
R ^a	Los Ranchos de Guadiamar	5	5	5 (5)	1	1
S25	Muñoz	6	6	7 (2)	1	1
S31	Minas del Castillo de las Guardas	3	4 (1)	4 (2)	1	1
S37	Valverde de Burguillos	5	6 (1)	13 (9)	1	1
S38	Valverde de Mérida	6	7 (1)	9 (3)	1	1.2 (1–2)
S41	Casar de Cáceres	4	13	14 (1)	3.25 (1–6)	1
S47	La Roca de la Sierra	4	4	10 (6)	1	1
S49	Robleda	5	17 (1)	20 (6)	2.8 (2–4)	1
S52	Guijo de Granadilla	7	7	9 (4)	1	1.25 (1–2)
S54	Casas de Millán	3	3	3 (3)	1	1
S55	Torre de Miguel Sesmero	5	5	8 (5)	1	1

^aPopulations surveyed by Leniaud *et al.* [9].

genotypes, the spermatheca of queens were dissected in Ringer's solution. Sperm content was released in a 10 µl volume of Ringer with extra care to avoid maternal tissue contamination. To extract DNA from minute sperm samples, we used a forensic QIAamp DNA micro kit (QIAGEN) with a final elution of 30 µl. We followed the manufacturer's recommended protocol for small sperm samples, including the addition of dithiothreitol (DTT) and carrier RNA in lysis buffers. DTT disrupts the disulfide bonds of sperm and permits sperm nuclear decondensation, while carrier RNA enhances the extraction yield.

(c) Genotyping

Virgin queens, mated queens and their mates (inferred from queens spermathecae and hereafter referred as 'males'), as well as a sample of six workers per colony were genotyped at 12 microsatellite loci. Haploid male genotypes could easily be determined from spermathecae as almost all queens were singly mated (see Results). Three loci (Cc11, Cc54 and Cc93) were developed for *Cataglyphis cursor* [20] and shown to amplify successfully in *C. hispanica* [9]. Nine new markers (Ch01, Ch05, Ch06, Ch08, Ch11, Ch12, Ch20, Ch22 and Ch23; table 2) were developed for *C. hispanica* using microsatellite-enriched shotgun pyrosequencing (Genoscreen, France). A mixture of DNA from 12 individuals originating from different populations was used to isolate microsatellite sequences [21]. A total of 5464 reads containing microsatellite repeat motifs were identified. Among these, the bioinformatics pipeline QDD [22] gave successful primer pairs for 317 loci. The MULTIPLEX MANAGER program [23] was used to select a set of 24 loci with dinucleotide repeats that could be amplified in the same polymerase chain reaction (PCR) conditions following a screening pipeline similar to the one used by Lepais & Bacles [24]. The amplification of the corresponding primer sets were tested on seven *C. hispanica* workers in standard

simplex PCR. Based on agarose gel visualization of PCR products, 17 out of 24 primers were validated. MULTIPLEX MANAGER predicted that nine out of these could be amplified in a 12-plex PCR including Cc11, Cc54 and Cc93. Forward primers were labelled with 6-FAM (Integrated DNA Technologies), VIC, PET or NED (Applied Biosystems) dyes. The 12-plex PCR reactions were carried out using a QIAGEN Type-it Microsatellite PCR kit (10 µl reactions following manufacturer's protocol with a 58°C annealing temperature). PCR products were separated on an ABI 3730 capillary sequencer using RadiantDy 632 internal size standard (BioVentures, Inc.). Allele scoring was carried out using GENEMAPPER software v. 3.5 (Applied Biosystems).

Because social hybridogenesis results in non-Mendelian patterns of inheritance, detection of null alleles was based on the analysis of genetic parentage of family groups [25]. False homozygotes are suspected if a worker is homozygous at an allele not detected in one of its parents, that is, if either of its parents fails to amplify at this locus or the queen is homozygous at a different allele than the one of its worker offspring (i.e. homozygote mismatch). Using this method, we did not find any evidence of null alleles in our sampling. All loci were highly polymorphic, showing between six and 24 alleles (table 2).

(d) Colony kin structure

First, we determined the number of queens per colony from field observations. Second, we estimated queen-mating frequency from the genotype of spermathecae contents. We considered the number of mates as equal to the maximum number of alleles at any locus. This method is straightforward because queens of *C. hispanica* typically mate singly (Leniaud *et al.* [9]; our present data). Third, we tested whether social hybridogenesis occurs across the species distribution range. To this aim, we considered three features of social

Table 2. Characteristics of 12 microsatellite loci in *C. hispanica*. (Primer names and sequences, fluorescent dye added to the 5' end of the forward primer, repeat motif type, number of observed alleles (N_A), corresponding size range of the amplified fragment and GenBank accession numbers are given for each locus.)

locus	primer (5'–3')	dye	motif	N_A	size range (bp)	GenBank
Ch01	F: GGAACGGATATCTGGAGCAA R: ATGTATGAAAATGCAGGCCG	NED	(GA) ₁₀	6	160–176	KC294415
Ch05	F: TTCTACAAAGCAAATGTCGTCCG R: ACGAGATGCGATCTTTGGTC	VIC	(AC) ₁₁	19	129–185	KC294416
Ch06	F: GAAGGAAGCTTTGCTGCTCA R: GCGGTATTACGGCATAACA	PET	(AG) ₉	20	162–212	KC294417
Ch08	F: GCTGATAATCGCGTCTGGAT R: CGACGTAAGAGGAACGTGA	NED	(TC) ₉	16	122–160	KC294418
Ch11	F: TTATTGTTAGACGCCACGAGC R: CGCGTTTTAAGTCGACCAAC	NED	(AG) ₉	16	222–258	KC294419
Ch12	F: ACAAGTTAATGTCGCCACC R: CGAGGCTTCATGAGACGATA	PET	(CT) ₁₁	16	90–144	KC294420
Ch20	F: CCCACGAAAATCAAAGTGG R: ACGAAACGAAAAGACGGAGG	PET	(GA) ₁₄	20	220–268	KC294421
Ch22	F: CGACGATCAAGCCGAAATAC R: AGCCTTTCATTCTGATGCT	VIC	(AC) ₁₁	12	282–304	KC294422
Ch23	F: CGGCATTACAAGTCATACAA R: CATCTTGCCTCGGTTTGATA	6-FAM	(GA) ₁₂	16	81–119	KC294423
Cc11 ^a	F: GATTGGCTTGGCGGTATC R: GGTCGCAAGATCAGAG	6-FAM	(GA) ₁₅	24	225–275	AY590645
Cc54 ^a	F: GAATTTGAATGGCTGATTGC R: ATGGTCGTTTGGCATAAAGG	6-FAM	(CT) ₁₇	30	155–279	AY590649
Cc93 ^a	F: CCTATCTATCTTGAACGCAGCTC R: TGTATTGAAGGACTCTTGATTCG	VIC	(TA) ₂₈	14	202–242	AY590659

^aLoci previously developed for *C. cursor* [20]; for those loci, motifs information is taken from published *C. cursor* sequences.

hybridogenesis [8,9]: (i) the pairing of reproductive individuals belonging to different genetic lineages, (ii) the production of hybrid workers by sexual reproduction, and (iii) the parthenogenetic production of queens. In each population sampled, we examined whether mating occurred between partners belonging to the same lineage or to different lineages based on the results of clustering analyses (see below). In addition, the mode of production of workers and new queens (i.e. parthenogenesis or sexual reproduction) was investigated using comparisons of parent–offspring genotypes. Sexually produced offspring bear one allele of their mother's mate at all loci, whereas parthenogenetically produced offspring bear alleles that can all be attributed to the queen.

(e) Diversity of hybridogenetic lineages across the species range

We used three complementary clustering approaches to determine the number of hybridogenetic lineages pairs across the species range: Bayesian clustering analyses, genetic-distance-based principal coordinates analysis (PCoA) and neighbour-joining tree based on shared allele distance. Our dataset consists of the genotypes of all available reproductives (the collected queens and their male mates inferred from the sperm stored in each queen's spermatheca), as well as of uncollected reproductives (inferred from workers genotypes; table 1). Because queens of *C. hispanica* are produced by parthenogenesis and disperse at short-range by

budding, there was a high probability of sampling queens with identical multilocus genotype in each population. In this case, only one queen per multilocus genotype was included in our analyses. Haploid male genotypes were encoded as diploid by doubling their alleles.

Bayesian clustering method implemented in STRUCTURE 2.3.3 [26] was used to determine the number of lineages (K) among reproductives. The program was run 10 times for each value of $K = 1–10$, with 100 000 Markov chain Monte Carlo iterations and a burn-in period of 40 000. Analyses were performed under the admixture model with independent allele frequencies and without prior population information. K -values that give consistent results between 10 runs are likely to represent real genetic structure. Therefore, the similarity coefficient among each pair of runs was calculated using the R-script STRUCTURE-sum-2009 [27]. The most probably number of genetic lineages was also investigated using the *ad hoc* δ - K method [28]. Graphical outputs were created with DISTRUCT [29]. Although the STRUCTURE model assumes Hardy–Weinberg and linkage equilibrium, it has been shown to perform well on data from parthenogenetic organisms, which violate these assumptions [30–32]. Moreover, INSTRUCT analyses, an alternative to STRUCTURE that drops the assumption of Hardy–Weinberg equilibrium [33], were performed with the same input parameters and returned exactly the same results as those of STRUCTURE (data not shown). Nevertheless, for cross-validation of STRUCTURE results, we carried out two model-free analyses. First, genetic-distance-based PCoA

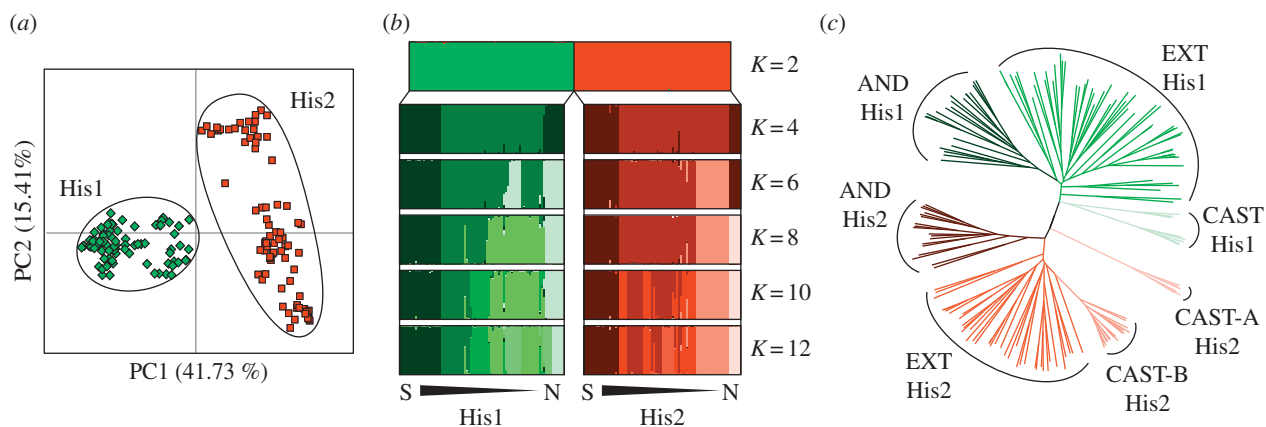


Figure 3. *Cataglyphis hispanica* consists of two hybridizing lineages. In this panel, colours distinguish lineages (His1 lineage, green; His2 lineage, red), as well as geographical variation within lineages (red and green values). Analyses are based on microsatellite data from 164 queens and males genotypes sampled across the 14 sites. (a) Plot of the first two axes from PCoA. The percentage of variation explained by each axis is indicated. (b) Population clustering analysis inferred with STRUCTURE, assuming two lineages and one to six within-lineage subpopulations. The highest probability run for each K is shown. (c) Neighbour-joining dendrogram based on allele sharing genetic distances (D_{AS}). In (b,c), within-lineages reproductives are ordered according to their sampling localities; data are given according to the south (S)–north (N) axis, which successively crosses Andalusia (AND), Extremadura (EXT) and Castile & León (CAS) regions.

was performed with GENALEX v. 6.41 [34]. Second, a neighbour-joining tree based on shared allele distance (D_{AS}) was constructed with POPULATIONS v. 1.2.31 [35].

To explore further the spatial structure of each genetic lineage, we performed a second batch of STRUCTURE on identified lineages. Also, we investigated isolation-by-distance within each lineage by plotting $[F_{st}/(1 - F_{st})]$ coefficients between pairs of populations against the \ln of the geographical distance [36]. Significance of correlation coefficient was assessed with a Mantel test as implemented in GENEPOP [37].

3. Results

(a) Colony kin structure

In 12 of 14 populations sampled, all colonies excavated were headed by a single queen ($n = 59$). Multiple-queen colonies were found in two populations (two colonies over four in population 49 and five over five in population 41; table 1); they were headed by two to six queens per colony. Sixty-eight queens collected over the whole sampling area were dissected for genetic analysis of their spermathecal content. Among these, 65 queens contained sperm from a unique multilocus haploid genotype, while three queens (4.4%) had no more than two male genotypes per spermatheca. Previous work (Leniaud *et al.* [9]) showed that the probability of non-detection of additional patrilineages owing to two fathers sharing the same alleles at all loci is lower than 0.04. These results indicate that single mating of queens (monoandry) is the rule in *C. hispanica*. Notably, all but five queens out of 68 (92.6%) had sperm from males of alternate lineage (see results below for lineages diversity). All five queens mated with males from their own lineage were found in multiple-matriline colonies.

Across the 14 populations sampled, workers were highly heterozygous (mean observed heterozygosity per locus \pm s.d. = 0.93 ± 0.06). All 408 workers analysed from the 68 colonies had inter-lineage genotypes resulting from hybrid crosses between two lineages. Not a single purebred worker was found, even in colonies with same-lineage mates. By contrast, neither virgin queens nor mated queens showed a genotype consistent with hybridization between two lineages.

All 88 mated queens collected (table 1) carried a pure-lineage genotype. Moreover, all of 22 virgin queens found ($n = 9$ colonies) showed a strictly identical genotype to the one of their mother's, indicating that they were produced by thelytokous parthenogenesis. Queens (mated and virgin) showed a high level of homozygosity (mean observed heterozygosity per locus \pm s.d. = 0.25 ± 0.18).

(b) Diversity of hybridogenetic lineages across the species range

We observed 164 different multilocus genotypes among a total of 210 reproductive individuals (i.e. the queens and their mates inferred from sperm or from workers genotypes, see Material and methods). The three clustering approaches showed strong lineage clustering (figure 3). On the PCoA (figure 3a), the genotypes formed two clusters distinguishable by their first coordinate (PC1), which accounted for 42% of total genetic variation. Individuals of both sexes and from all collecting sites were found in each cluster. The neighbour-joining tree reflected the same assignments, but each cluster had further regional structuring corresponding to biogeographic zones (Castile & León, Extremadura and Andalusia, figure 3c). Bayesian analyses using STRUCTURE confirmed these results (figure 3b; see also the electronic supplementary material, figure S1). Likelihood values increased with the number of groups (K), but the second-order likelihood (δK) was much higher for $K = 2$. Moreover, all runs performed with $K = 2$ showed identical results, while higher values of K gave inconsistent results. Separate analyses resulted in geographical structuring as seen in the neighbour-joining tree, although the definition of clusters was unstable over runs according to similarity coefficients (see the electronic supplementary material, figure S1). For each of the two groups, there was significant correlation between population genetic differentiation and the geographical distance (Mantel test: His1, $r = 0.37$, $p < 0.00001$; His2, $r = 0.47$, $p < 0.00001$).

Altogether, these results indicate that the reproductive individuals of *C. hispanica* belong to two lineages that are sympatric across the entire range of the species. These

lineages correspond to the single pair His1/His2 previously described in Andalusian populations [9]. The two interbreeding lineages formed highly divergent gene pools: nine out of 12 loci (Cc11, Cc54, Cc93, Ch01, Ch05, Ch08, Ch12, Ch20 and Ch23) had distinctly different allele size distributions, with 63–97% of alleles being diagnostic of the lineage per locus (see the electronic supplementary material, figure S2). Allele size distributions were overlapping between the lineages for the remaining three loci (Ch06, Ch11 and Ch22).

4. Discussion

Our results show that social hybridogenesis is a major feature of the reproductive system of the desert ant *C. hispanica*. Social hybridogenesis occurs in all populations sampled along a 400 km transect crossing the whole distribution range of the species. In the 68 colonies studied: (i) the male and female sexuals are pure-lineage individuals, consistent with their parthenogenetic production; (ii) the queens and their mates generally belong to different genetic lineages; and (iii) all workers arise from hybrid crosses between the lineages. Remarkably, our data show that all workers are hybrids of the same two highly divergent genetic lineages across the 14 sampled populations

A single queen usually heads each colony, but populations with multiple-queen colonies do exist (two to six queens per colony). Recently, social hybridogenesis was reported in multiple-queen colonies of *C. velox* and *C. mauritanica*, indicating that monogyny is not a prerequisite for the evolution of hybridogenesis in *Cataglyphis* ants [8]. Queens of *C. hispanica* are mated once (rarely twice) with male(s) originating from the alternative lineage of their own and use sperm to produce hybrid workers by sexual reproduction. Rare intra-lineage mated queens were found (five out of 68) in multiple-queen populations. However, these queens seem to usurp multiple-queen colonies as not a single pure-lineage worker genotype was found, indicating that they do not contribute to worker production. By contrast with workers, daughter queens have identical multilocus genotypes to the one of their mother indicating that they arise from thelytokous parthenogenesis. Previous studies showed that thelytoky proceeds by automixis with central fusion in other *Cataglyphis* species, including *C. velox* and *C. mauritanica* [8,38,39]. Whether thelytokous parthenogenesis in *C. hispanica* also proceeds by automixis awaits further study.

All queens from single-queen colonies were found mated with males originating from the co-occurring alternative lineage. Two non-mutually exclusive hypotheses may explain this result. First, mating between sexual partners from the same lineage may be avoided through behavioural constraints, for example chemical recognition (pre-zygotic isolation) [40,41]. Alternatively, intra-lineage mating may occur but have a low selective value owing, for instance, to queens dying prematurely as they cannot produce workers (post-zygotic isolation). A mechanism of assortative mating is likely to exist owing to strong selection pressures on both queens (which rarely mate more than once) and colonies (which produce very few reproductives each year). Yet, the rare occurrence of intra-lineage mated queens in multiple-queen populations suggests that pre-zygotic isolation of lineages is not complete and that post-zygotic mechanisms must be involved. In line with this hypothesis, repeated laboratory experiments showed

that eggs laid by intra-lineage mated queens fail to develop into workers (H. Darras 2013, unpublished data). In multiple-queen colonies, post-zygotic isolation may be relaxed as intra-lineage mated queens may be able to survive as parasites producing only reproductive individuals.

Our data indicate that social hybridogenesis allows the maintenance over time and across a large geographical scale of highly divergent genetic lineages, despite their constant hybridization. Two distinct genetic lineages (His1/His2) were previously identified in four southern populations of *C. hispanica* separated by less than 50 km [9]. Our large-scale survey shows that these two lineages are spread across the whole distribution of the species. Hybridizing lineage pairs formed by queens and their mates in each population are all derived from a single lineage pair His1/His2. This pair consists of two highly divergent gene pools that formed well-defined clusters in our analyses (figure 3). Although one may not completely exclude the existence of alternative lineages over the distribution area, such lineages should be very rare and must be considered as anecdotal in the present context. Suppose that there are three genetic lineages with binomial distribution in the species. With a sample size of 164 multilocus genotypes and assuming a significance level of $\alpha = 0.05$, a third genetic lineage should occur at a maximum frequency of 0.018; thus, there are 55 times more individuals belonging to the lineages His1 and His2 than to a putative lineage His3. Our sampling does not allow determination of the ratio of the two lineages in *C. hispanica*. Skewed lineage ratios have been reported in dependent-lineage populations of *Pogonomyrmex* harvester ants, possibly owing to differences between lineages in sex ratio allocation [42].

The high percentage of diagnostic alleles in nine out of 12 loci surveyed supports a lack of current gene flow between the His1/His2 lineages. This interpretation requires some caution, however, because substantial gene flow can occur on restricted genome parts of lineages [43]. Introgression through rare hybrid queen production may therefore remain undetected while using only a handful of marker loci. Rare development of queens from hybrid eggs has been reported in other ant systems relying on hybridization between divergent lineages [18,44–46]. Though, not a single *C. hispanica* hybrid queen has been found so far ([9]; this study). Future linkage studies using a larger number of nuclear markers [46] will help to determine whether His1/His2 have achieved complete genetic isolation or whether some parts of their genome are leaky.

The two lineages exhibit patterns of isolation by distance and similar genetic substructure (figure 3*b,c*). This was most notable on the neighbour-joining tree, which separates three to four geographical zones for each lineage that reflect major mountain chain and river barriers to gene flow between Andalusia, Extremadura and Castile & León regions. Strong genetic viscosity is expected in *C. hispanica* because females establish new colonies at a walking distance of their natal nest with the help of worker cohorts, resulting in the formation of clonal patches [9]. Males of *C. hispanica* disperse by flight, but do not contribute to the spatial genetic structure, as they do not transmit their genes to the reproductive offspring. In *C. hispanica*, the persistence of social hybridogenesis requires the maintenance of viable hybrid workers. As sympatric lineages do not recombine, the two members of a lineage pair must coevolve to maintain a worker caste. Ultimately, the long-term isolation of geographically co-adapted lineages may lead to the speciation of sublineages belonging to the His1/His2 pair. New lineage formation through geographical

isolation or population bottlenecks was previously suggested in dependent-lineage populations of *Pogonomyrmex* ants. A single ancestral lineage pair belonging to the *P. barbatus/rugosus* complex was assumed to give rise to at least three pairs of lineages that currently overlap somewhat (the pairs F, G and H [18]), although this subdivision of the ancestral pair has been questioned recently [46]. Our data do not allow testing of this issue in *C. hispanica*. Experimental crossing between partners belonging to alternative genetic lineages and from different populations would allow us to determine whether coevolution has taken place to an extent where geographically distant partners have become incompatible.

The origin and evolution of hybridogenetic lineages in *C. hispanica* remains uncertain. Schwander & Keller [47] proposed that lineage pairs are of recent origin. They expect lineages to be short-lived because queens should be under selection to stop the production of males, which do not transmit their genes to the reproductive offspring. Assuming that hybridogenetic lineages are dead-ends, they argued that only minor evolutionary steps are required to switch back and forth from social hybridogenesis to facultative parthenogenesis, a probable ancestral feature of the genus *Cataglyphis*. According to this scenario, social hybridogenesis would be a transitory system that regularly evolved in the genus. Alternatively, social hybridogenesis could have ancient genomic roots that have locked hybridogenetic *Cataglyphis* in this reproductive system since its origin. This could take place, for example, if social hybridogenesis is controlled by a supergene. Supergenes are clusters of tightly linked loci inherited as a single unit; they maintain specific combinations of alleles. They have been described for a long time in a diverse array of organisms such as plants or mimic insects [48,49]. A supergene was recently reported to determine social organization in the fire ant *Solenopsis invicta*. This species exhibits two social forms, one with single-queen colonies and one with multiple-queen colonies that differ in many fundamental reproductive and

social traits [50]. The two forms can be predicted based on genotypes at the Gp-9 gene, with queens from single-queen colonies being homozygous BB and queens from multiple-queen colonies being heterozygous Bb [51]. Phylogenetic analyses of Gp-9 sequences showed that the two allelic variants, B and b, are conserved in four species known to possess a polymorphism in social organization similar to the one found in *S. invicta*. In these species, queens from single-queen colonies only possess B-like alleles, whereas queens from multiple-queen colonies also harbour b-like alleles [51,52]. This suggests that the origin of b-like alleles inducing polygyny preceded the origin of these five species. The Gp-9 gene is part of the fire ant supergene, which corresponds to about half a chromosome where recombination events are completely suppressed between the two forms [53]. Several authors have suggested that supergenes affecting social organization may have evolved in other social insects as well [53,54]. The hypothesis of a biallelic supergene similar to the two variants at Gp-9 indeed is an appealing scenario to account for the evolution of hybridogenesis in *Cataglyphis*. The two forms of a supergene would account for the occurrence of only two genetic lineages in all populations of *C. hispanica*, *C. velox* and *C. mauritanica* sampled so far. The last common ancestor of these three sister species is thought to have lived at least 5.3 Ma [8,55]. A shared origin of social hybridogenesis would therefore support an ancient origin predating their speciation.

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Data accessibility. DNA sequences: GenBank accessions KC294415–KC294423. Microsatellite genotype data for all individuals are available in the electronic supplementary material, Genotypes.txt.

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