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1 A putatively new ant species from the *Cataglyphis cursor* group displays low
2 levels of polyandry with standard sexual reproduction

3
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21 Running title: Population and colony genetic structure of a putative new ant species

22 Keywords: sociogenetic organisation, relatedness, polyandry, thelytoky, ants

23

24 ABSTRACT

25 How social organisation (number of queens and mating partners) and reproductive system (use of
26 thelytoky vs sex) evolved in ants and how they interplay is still far from clear. The *Cataglyphis* desert
27 ants represent a fascinating group as it harbours variation in both levels of polyandry and the use of
28 thelytoky, with polyandry and sexual reproduction probably being the ancestral state. In this study,
29 we compared the colony and population genetic structure of two populations of the *Cataglyphis*
30 *cursor* group distributed in a remote small geographic area in central Spain with two closest
31 geographic species *C. piliscapa* and *C. cursor*. We found that the two populations are genetically
32 isolated from *C. piliscapa* and *C. cursor*. However, the population genetic diversity was like *C.*
33 *piliscapa* and *C. cursor*. We observed a lower level of polyandry in *C. cursor* from Spain compared to
34 *C. piliscapa* and *C. cursor*. We found no evidence that *C. cursor* queens from Spain reproduce
35 thelytokously. This supports the hypothesis that thelytoky is disfavored in systems with lower levels
36 of polyandry. The reversion from polyandry towards monandry has rarely been observed in ants. We
37 present multiple hypotheses that could explain this phenomenon. We raise the possibility that these
38 remote populations belong to a putatively new species. However, this requires a careful taxonomic
39 investigation. Our study confirms the lability of the reproductive system in this group and provides an
40 exciting system to investigate the coevolution of polyandry and thelytoky.

41

42 INTRODUCTION

43 The ancestral state of the social Hymenoptera is supposed to be simple with a single reproductive
44 queen (monogyny) mated with a single male (monandry) and the queen reproduces sexually (Hughes
45 et al. 2008; Bourke 2011). This social structure provides a high inclusive fitness benefit to helpers
46 because of the inflated relatedness among colony members. In spite of this, many species have
47 evolved polygyny (multiple queens) and polyandry (multiple mates)(Crozier et al. 1996; Hughes et al.
48 2008; Bourke 2011). Further, thelytokous reproduction (parthenogenetic and diploid production of
49 females) has evolved multiple times independently (Wenseleers and Van Oystaeyen 2011; Rabeling
50 and Kronauer 2013; Goudie and Oldroyd 2018; Kuhn et al. 2020). Polygyny and polyandry typically
51 reduce relatedness and decrease the inclusive fitness benefits of helping. In contrast, thelytoky
52 increases relatedness. However, its consequences on the genetic architecture of the colony varies
53 depending on the caste produced by thelytoky (Greeff 1997; Wenseleers and Van Oystaeyen 2011;
54 Goudie and Oldroyd 2018). The factors driving the evolution of breeding systems (number of
55 breeders in a group), mating systems (number of mating partners) and reproductive systems (the
56 genetic system underlying the transmission of genes from one generation to the next) and the
57 interplay between these systems are poorly understood. If the evolution and relationship between
58 polygyny and polyandry has received attention, with a negative association being first predicted by
59 Keller and Reeve (1994) and later supported in social Hymenoptera (Kronauer and Boomsma 2007;
60 Hughes et al. 2008), little is known about the relationship between polyandry and/or polygyny and
61 the evolution of thelytoky (Wenseleers and Van Oystaeyen 2011).

62 *Cataglyphis* desert ants represent a fascinating group as the genus has evolved a plethora of
63 breeding, mating and reproductive systems. For example, some species have very high levels of
64 polyandry and/or others have evolved peculiar forms of conditional thelytoky in which the queen
65 uses sexual reproduction for worker production but thelytokous reproduction for new queen
66 production (Pearcy et al. 2004; Doums et al. 2013). In some species, the queen mates with distinct

67 lineages of males to produce the worker caste and reproduces thelytokously to produce the queen
68 caste. This system is called “clonal social hybridogenesis” (Leniaud et al. 2012; Darras et al. 2014;
69 Kuhn et al. 2020). *Cataglyphis* genus comprises approximately one hundred species that are
70 distributed across the arid regions of the Old World. *Cataglyphis* is characterised by a strict diurnal
71 activity and by being highly thermophilic (Lenoir et al. 2009; Boulay et al. 2017). The ancestor of this
72 genus was most likely monogynous and polyandrous, but the use of thelytoky is not clear in the
73 ancestor (Aron et al. 2016). Currently, conditional thelytokous reproduction has been demonstrated
74 by the queen in 11 species from two of the seven main monophyletic groups. This suggests that
75 thelytoky has evolved multiple times independently and within each group (Kuhn et al. 2020).
76 Workers can also reproduce thelytokously in eight species. This typically occurs in queenless
77 conditions (Aron et al. 2016; Table 1). The *C. cursor* group is unusual because in the five species
78 where thelytoky has been assessed, the queen was conditionally thelytokous. Further, four of these
79 species exhibited ‘clonal social hybridogenesis’ (Kuhn et al. 2020). However, intraspecific variation
80 was also observed. In *C. aenescens*, evidence of thelytoky was found in one population (Kuhn et al.
81 2020) but not in another (Cronin et al. 2016). Variable rates of thelytokous and sexual queen
82 production have also been observed across populations of *C. piliscapa* (Pearcy et al. 2004; Doums et
83 al. 2013).

84 Since the number of mating partners and the mode of reproduction (thelytokous and sexual
85 reproduction) affect genetic relatedness among individuals, their evolution may not be independent.
86 From a kinship perspective, thelytokous queen production by the queen, as in *C. piliscapa*, is more
87 likely to evolve in highly polyandrous systems (Doums and Monnin 2020). When the relatedness is
88 lower than 0.5 among workers, the genetic interest of workers and queens converge. Workers should
89 favor rearing thelytokously produced queens by the mother queen because they are more closely
90 related ($r = 0.5$) to thelytokously produced queens compared to sexually produced queens ($r <$
91 0.5)(Wenseleers and Van Oystaeyen 2011). In monandrous systems, workers should favor rearing
92 sexually produced queens given that workers are more closely related to the next queen ($r = 0.75$)

93 compared to thelytokously produced queens ($r = 0.5$). Conversely, the evolution of social
94 hybridogenesis can potentially reduce the benefits of multiple mating because the high levels of
95 worker heterozygosity is already obtained by queens mating with males from different genetic
96 lineages (Aron et al. 2016). Studying new populations and species from this unique group is
97 necessary to assess the relationship between polyandry and thelytoky.

98 In this study, we assessed the population and colony genetic structure of two populations from the
99 *Cataglyphis cursor* group in a geographical area in Central Spain. We characterised the population
100 and colony genetic structure to infer the degree of polyandry and the presence of thelytoky. This
101 area is restricted to approximately 10 000 km² and is isolated from the populations of *C. piliscapa* in
102 Southern France (Fig. 1 and Fig. S1). *C. piliscapa* use conditional thelytoky with queen inbreeding
103 without social hybridogenesis (Pearcy et al. 2004; Doums et al. 2013). In contrast, a closely related
104 species from the South of Italy, *C. italica*, employs thelytoky with social hybridogenesis (Kuhn et al.
105 2020). Clonal social hybridogenesis is associated with extremely high levels of heterozygosity in
106 workers due to queens mating with a distinct male genetic lineage that hybridise to produce workers
107 (Leniaud et al. 2012; Eyer et al. 2013). For simplicity, we will refer to the two populations as *C. cursor*
108 from Spain. The cuticular hydrocarbons profiles of workers from this region were distinct from those
109 of *C. piliscapa* and *C. cursor* suggesting that they could belong to a different species (Nowbahari et al.
110 1990). Note that most of the systematics of the *cursor* group is unresolved (Aron et al. 2016). Here,
111 we assessed the genetic relationship between *C. cursor* from Spain to the geographically closest
112 species based on mtDNA (for *C. piliscapa*, *C. cursor* and *C. italica*) and on microsatellite markers (for
113 *C. piliscapa* and *C. cursor*). We also compared the patterns of population genetic structure of *C.*
114 *cursor* from Spain (isolation by distance and genetic diversity) to *C. piliscapa* and *C. cursor*.
115 Additionally, we characterised and compared the level of polyandry to populations of *C. piliscapa*.
116 Finally, we directly tested for the presence of thelytoky by genotyping young queens, and indirectly
117 by estimating (by maximum likelihood) the number of successive generations of thelytoky for each
118 queen.

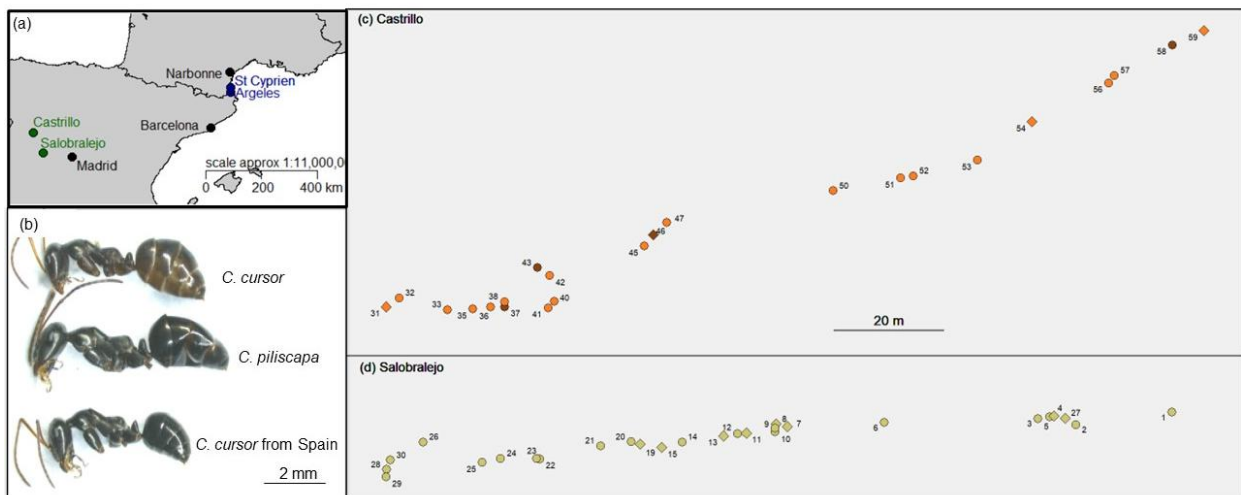
119

120

121 **Materials and methods**

122 *Sampling*

123 We sampled fifty colonies in two separate locations in Spain. We sampled 23 colonies in Castrillo de
124 Guareña (41.2301° N, 5.3253° W) in April 2014 and 27 colonies in Salobrelejo (40.5860° N, 4.9046°
125 W) in July 2015. The two locations are separated by approximately 80 kilometers (Fig. 1, Table S1).
126 We recorded the GPS coordinates for each colony (the coordinates are given in Table S1). We
127 collected workers closed to the nest entrance as it was not possible to excavate the entire colony due
128 to the soil condition. In Salobrelejo, we collected nine gynes at the nest entrance from three
129 colonies. This allowed us to directly estimate the use of thelytoky. We placed collected individuals in
130 95% Ethanol for genomic sequencing (with 5% Tris-EDTA).



131

132 **Fig. 1** Sampling sites and colony mapping of *C. cursor* from Spain and France. (a) The two populations
133 of *C. cursor* in Spain (green) are separated by approximately 80 kilometers. We used two populations
134 of *C. piliscapa* (blue) to compare the genetic structure of colonies. (b) Photograph of workers from *C.*
135 *cursor*, *C. piliscapa* and *C. cursor* from Spain. Geographic mapping of colonies from (c) Salobrelejo and
136 (d) Castrillo. Each circle represents a colony where a single worker was analysed. Each diamond

137 represents a colony where more than one individual was analysed. The colors represent the three
138 different haplotypes we found (one in Salobralajo and two in Castrillo). The numbers refer to each
139 colonies ID (see Table S1).

140

141 *Genetic analysis*

142 All analyses were conducted using R v.3.6.0 (R Core Team 2019), except when otherwise stated. We
143 produced figures with the library 'ggplot2' (Wickham et al. 2023).

144 **Mitochondrial DNA.** We extracted total genomic DNA from the heads of workers using the Qiagen
145 extraction kit (DNA Easy blood and tissue kit). We sequenced a portion (555 bp) of the cytochrome
146 oxidase I (COI) locus from a single worker from each colony following the protocol of Clémencet et al.
147 (2005). We also genotyped five individuals of *C. rosenhaueri* as an outgroup for the mtDNA. This
148 species does not belong to the *cursor* group. We sent the PCR products to GENOSCREEN (Lille,
149 France) for Sanger sequencing. We performed base calling and sequence reconciliation with BioEdit
150 (Hall 1999). We aligned sequences with the MUSCLE algorithm in MEGA version 6 (Tamura et al.
151 2013).

152 We combined our data with those of Eyer et al. (2022), which included the same mtDNA fragment
153 from 52 workers from 26 locations (a single worker from two colonies per location) that were
154 sampled in southern France and northern Spain for *C. cursor* and *C. piliscapa* and two individuals of *C.*
155 *italica* (see Fig. 1 from Eyer et al. (2022)). Using the distinct haplotypes found in *C. cursor*, *C. piliscapa*
156 and *C. italica* from Eyer et al. (2022) and those of our study (three for *C. cursor* from Spain and two
157 for *C. rosenhaueri*), we reconstructed the phylogenetic relationships among haplotypes using the
158 program MrBayes v.3.1.2 with Bayesian Inference (BI)(Ronquist et al. 2012). To determine the
159 substitution model with the best fit, we used PartitionFinder v. 1.1.1 (Lanfear et al., 2012) and
160 subsequently chose the 'GTR' substitution model with a gamma-distributed rate variation across sites
161 and a proportion of invariable sites for our evolutionary model. We estimated tree topology and

162 node support using a Metropolis-coupled Markov chain Monte Carlo sampling approach. Here, we
163 used two runs and four chains (three hot, one cold) running for 1×10^7 generations. We saved one
164 tree every 1000 generations. We assessed convergence by examining the standard deviation of the
165 split frequencies between the two runs (< 0.01) and the Potential Scale Reduction Factor diagnostic.
166 We kept this diagnostic close to 1. We classified the first 1000 trees from each run as burn-in. We
167 generated a majority-rule consensus tree from the 9000 remaining trees. We visualised the
168 consensus tree with the posterior probabilities using FigTree (Rambaut 2010). We used the two
169 haplotypes of *C. rosenhaueri* to root the tree.

170 **Microsatellite makers.** From our 50 colonies, we genotyped a single worker per colony from 37
171 colonies (Table S1). For the 13 remaining colonies, we collected and genotyped 114 workers (86
172 workers from nine colonies in Salobralejo and 28 workers from four colonies in Castrillo) to assess
173 the genetic structure of colonies (Table 1). We genotyped nine gynes from three colonies in
174 Salobralejo (Table 1). The individuals were screened for fifteen microsatellite loci (thirteen loci from
175 Eyer et al. (2022) and two supplementary loci (L76 and L3653)). We report loci characteristics and
176 PCR conditions in Table S2. We removed the L26 locus due to amplification failures in nine individuals
177 and a significant Hardy-Weinberg disequilibrium ($P < 00001$) for both sites. Collectively, these results
178 suggest the presence of null alleles at this locus. The genetic analyses on all 14 loci were conducted in
179 parallel with those performed by Eyer et al. (2022) avoiding allelic calling bias between the
180 populations analysed.

181 **Population genetic structure.** In colonies where we sampled more than one worker, we randomly
182 chose a single individual. We paid attention to randomly select workers with no missing data. We
183 obtained 27 workers from Salobralejo and 23 from Castrillo. We tested for linkage disequilibrium
184 between loci and Hardy-Weinberg equilibrium within each location using 'exact tests in Genepop
185 (Rousset 2008). We estimated allelic richness using the rarefaction method implemented in the
186 package 'PopGenReport' (Gruber and Adamack 2022) allowing to control for different sample sizes

187 (El Mousadik and Petit 1996). We calculated Nei gene diversity for each locus and location with
188 'PopGenReport'. We performed a Mantel test in Genepop to assess the pattern of isolation by
189 distance between pairs of individuals within each location. We used the \hat{d} genetic distance statistic
190 between individuals (Rousset 2008) and the linear geographic distance. We performed 1000
191 permutations for the Mantel test. We combined the data from Eyer et al. (2022) and our samples to
192 estimate the degree of isolation from the other populations of *C. cursor* and *C. piliscapa*. We
193 estimated F_{st} using an 'exact test' in GenePop. For comparisons with the *C. piliscapa* and *C. cursor*
194 populations analysed by Eyer et al. (2022), we also calculated the matrix of Hedrick G_{st} between each
195 pair of locations (Hedrick 2005) with 'PopgenReport'. This corrects the F_{st} value for the observed
196 within population diversity. We visualised the pairwise distance matrix (G_{st}) between each pair of
197 locations using a non-parametric multidimensional scaling plot (NMDS) using the 'MASS v 7.3-59'
198 library in R (Ripley et al. 2023).

199 **Colony genetic structure.** We used COLONY (Jones and Wang 2010) to identify full and half-sister
200 clusters using a maximum likelihood approach with no prior knowledge of an individual's nest of
201 origin. We included the colonies with a single worker to obtain a better estimate of allele
202 frequencies. We removed individuals with three or more missing loci from the data set. A visual
203 check of the output confirmed that COLONY identified clusters of individuals belonging to the same
204 colony and sharing the same mother except in seven colonies in which one individual was assigned to
205 a different mother. We visually checked that these seven individuals always had at least three loci
206 that did not share any alleles with the putative mother of the colony inferred from the other
207 individuals (see Results section). We estimated the effective mating frequency ($ke3$) with the formula
208 from Nielsen et al. (2003). We estimated the probability of not detecting a patriline which can occur
209 when two males have the same allelic combination at a polymorphic loci by chance. At the
210 population level, this probability is given by: $P_{nondetection} = \prod_l (\sum p_i^2)$ where p_i denotes the
211 allele frequencies at each i loci (Boomsma and Ratnieks 1996). We compared the patrilines in
212 Castrillo and Salobralejo with those observed in *C. piliscapa* from Doums et al. (2013, 2018). We

213 included the number of patriline and workers per patriline surveyed in 15 colonies from Argeles in
214 southern France. However, Doums et al. (2013, 2018) analysed a greater number of workers per
215 colony in *C. piliscapa*. Therefore, we performed a resampling simulation to estimate the level of
216 polyandry in *C. piliscapa* based on our sampling of *C. cursor* from Spain. For each simulation, we
217 randomly sampled without replacement the same number of colonies with the same sample size per
218 colony as the one observed in our location of interest (Salobralejo or Castrillo). We estimated the
219 mean number of patrilines observed in this subsample. We carried out 1000 simulations for each
220 location and obtained the 95% confidence interval (CI) by selecting the threshold value defining the
221 2.5% and 97.5% of the distribution of simulated values.

222 We estimated relatedness among females within colonies using COANCESTRY. These estimates were
223 based on the population allelic frequency obtained with COLONY. The Queller and Goodnight
224 estimator of relatedness was used and averaged by colony (Queller and Goodnight 1989). The colony
225 mean relatedness was then compared with the distribution of pairwise relatedness expected under
226 either the full-sister ($R = 0.75$) or the unrelated assumption ($R = 0$). The expected relatedness
227 distribution was calculated based on a simulation of 1000 individuals using the population allelic
228 frequency and the same frequency of missing data per locus as the one observed in the dataset. The
229 simulation was performed for each site using COANCESTRY.

230 **Use of thelytoky.** We directly assessed the use of thelytoky by comparing the genotypes of the nine
231 gynes (Table 2). To indirectly assess the use of thelytoky in the two locations, we inferred the
232 genotype of the mother queen from the genotype of the workers in the same colony using COLONY.
233 For one locus, less than five workers could be genotyped. Therefore, we did not infer the maternal
234 genotype at this locus. From the 13 putative genotypes of the mother queen and all loci, we
235 identified 17 situations with more than one possible genotype. For all these situations except one,
236 the two possible genotypes at one locus were homozygous but for a different allele (all offspring
237 were heterozygous for the same alleles). In one situation where the state of zygosity could not be

238 clearly determined, this site was most likely to be homozygous with a probability of 0.922 and was
239 therefore considered as homozygous.

240 We used a maximum likelihood approach to estimate the expected number of generations of
241 thelytoky for each inferred mother and the gynes. The full method for obtaining the maximum
242 likelihood estimates is given in the Supplementary materials. We compared these estimates with
243 those based on genotyped queens and gynes from Argeles (102) and St Cyprien (143) (Doums et al.
244 2013). Three pieces of data were entered into the model: (i) a matrix of queens by locus where 0 is
245 heterozygous and 1 is homozygous, (ii) for each locus, the number of heterozygous workers over the
246 total number of workers for each locality (to estimate the probability of being homozygous for that
247 locus, assuming sexual reproduction) and (iii) for each locus, the number of homozygous daughters
248 (gynes) produced by a heterozygous mother queen following thelytokous reproduction (to estimate
249 the loss of heterozygosity (LOH)). LOH represents the transition rate from heterozygosity to
250 homozygosity during thelytokous reproduction at a given locus. This transition rate was based on
251 data from Doums et al. (2013) and unpublished data to improve parameter estimation (Table S2). We
252 assumed the same LOH rate per locus in *C. cursor* from Spain than in *C. piliscapa*. The LOH rate
253 depends on the type of thelytokous reproduction (central or terminal fusion) and the physical
254 distance to the centromere (Svendsen et al. 2015) and is therefore not expected to vary between
255 closely related species.

256 We also used this maximum likelihood method to estimate the number of successive generations of
257 thelytoky (SGT) from the two already studied populations of *C. piliscapa* mentioned above (Argeles
258 and St Cyprien). We compared these estimates to our two Spanish populations. We used non-
259 parametric Wilcoxon rank tests to assess differences between the populations. We compared the
260 SGT estimates of *C. cursor* from Spain with (i) those of *C. piliscapa* obtained from mother queens (the
261 two populations were pooled as there were no differences observed in the use of thelytoky) (Doums
262 et al. 2013) (ii) the SGT estimates of *C. piliscapa* obtained from gynes known to be produced by

263 thelytokous reproduction and (iii) from gynes known to be sexually produced. In the second and third
264 comparisons, we used the SGT estimates averaged by colony.

265

266 **Results**

267 *mtDNA diversity and phylogenetic relationships*

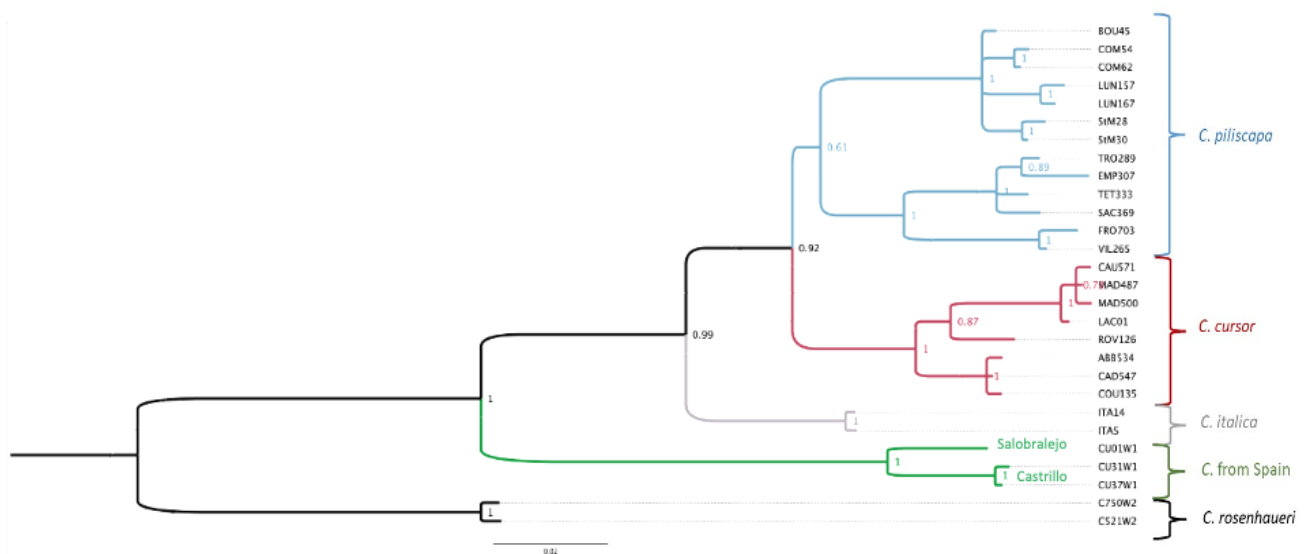
268 Out of the 50 sequenced workers from the two Spanish localities, we identified three different
269 mitochondrial haplotypes, one in Salobralejo and two in Castrillo (one haplotype was represented in
270 83% of Castrillo individuals) (Fig. 1c, d). The two Castrillo haplotypes differed by a single mutation
271 whereas the haplotype found in Salobralejo differed by 23 mutations compared to the Castrillo
272 haplotypes (p distance = 0.041). The low level of diversity observed within each location supports
273 previous findings in *C. cursor* and *C. piliscapa* (Clémencet et al. 2005; Eyer et al. 2022). This low
274 diversity prevented the testing of isolation by distance. However, the two haplotypes did not cluster
275 in Castrillo: the four colonies with the low frequency haplotype were scattered across the map (dark
276 brown colonies in Fig. 1C). The phylogenetic tree grouped the haplotypes according to species (Fig.
277 2). We found that based on phylogenetic distance, *C. cursor* from Spain was equally related to both
278 sister species *C. cursor* and *C. piliscapa* (mean p distance = 0.12 for both species) as to *C. italica*
279 sampled in southern Italy (mean p distance = 0.11) (Fig. 2).

280

281 *Population genetic analysis*

282 From the 144 linkage disequilibrium tests, only seven were significant with p -values all greater than
283 0.016. These seven tests were not statistically significant after performing Bonferroni corrections
284 (Table S3). HW equilibrium tests were not significant after Bonferroni corrections (Table S4). In social
285 hybridogenetic species, workers are produced by sexual reproduction from two genetically distinct
286 lineages and harbour a high level of heterozygosity by locus. This system promotes heterozygosity at

287 each locus and lead to a significant deviation from HW (Leniaud et al. 2012; Eyer et al. 2013). The
 288 absence of HW deviation in our workers therefore suggests the absence of this mode of reproduction
 289 in the two populations studied. One microsatellite locus (L46) was not polymorphic at both locations.
 290 Allelic richness at each location and locus ranged from 1 to 10.44 (mean \pm SD = 4.15 ± 2.29) and gene
 291 diversity from 0 to 0.92 (mean \pm SD = 0.63 ± 0.24) (Table S5). The allelic richness was similar between
 292 the two locations (Table S5, Wilcoxon paired test = 39, $df = 13$, $p = 0.67$) as well as the gene diversity
 293 (Table S6, Wilcoxon test paired by locus = 49.5, $df = 13$, $p = 0.81$). There was no significant difference
 294 between the mean allelic richness in *C. cursor* from Spain and the mean allelic richness in *C. piliscapa*
 295 (Wilcoxon paired test = 40, $df = 12$, $p = 0.97$) or in *C. cursor* (Wilcoxon paired test = 20, $df = 12$, $p =$
 296 0.15). There was no significant difference between the mean gene diversity in *C. cursor* from Spain
 297 and the mean gene diversity in *C. piliscapa* (Wilcoxon paired test = 24, $df = 12$, $p = 0.27$) or in *C.*
 298 *cursor* (Wilcoxon paired test = 46, $df = 12$, $p = 0.62$). Therefore, the two locations had a similar
 299 genetic diversity to those in *C. piliscapa* and *C. cursor* in France.



300

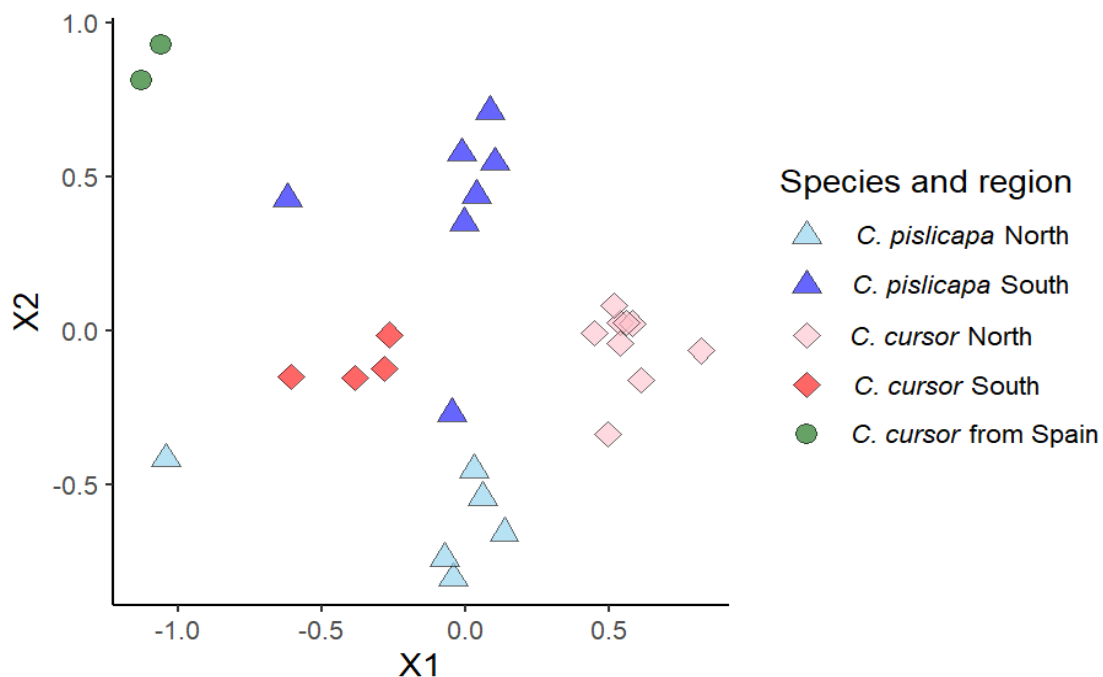
301 **Fig. 2** Bayesian phylogenetic tree of *C. cursor* group relationships for the studied species based on the
 302 COI gene. The support values on the nodes correspond to posterior probabilities. The branches are

303 colored according to the geographical distribution of the haplotypes. *C. rosenhaueri* was used as an
304 outgroup of the *C. cursor* group.

305

306 We found significant population differentiation between Castrillo and Salobralejo ($F_{st} = 0.14 \pm 0.11$,
307 exact test of genic differentiation $p < 0.0001$). The NMDS plot separated the two locations of *C.*
308 *cursor* from Spain from those of *C. piliscapa* and *C. cursor* (Fig. 3). These results were similar to the
309 findings from the mtDNA with the two populations of *C. cursor* from Spain also clustering together.
310 We found no evidence for significant positive correlations between genetic distance and
311 geographical distance between pairs of individuals in both locations (Mantel exact test, Salobralejo: p
312 = 0.27, Castrillo: $p = 0.63$).

313



314

315 **Fig. 3** NMDS plot over the first two dimensions (X1 and X2) based on Hedrick's G_{st} from the
316 microsatellite data. We estimated the Hedrick's G_{st} between each pair of locations (dots in the
317 figure). The species are represented the same way as Eyer et al. (2022) for *C. piliscapa* (triangle, light

318 and dark blue for the North region and South region respectively) and *C. cursor* (square for *C. cursor*,
319 pink and red for the North and South region respectively). The two locations of *C. cursor* from Spain
320 are represented green circles. The stress value of the NMDS was 17.83%.

321

322 *Colony genetic structure*

323 From the 13 colonies analysed, seven colonies had an individual with at least three loci that did not
324 share any alleles with the inferred putative mother (called later foreign individuals). We inferred the
325 putative mother of the colony from the genotype of the other members of the colony (Table 1).
326 Polygyny could explain the presence of these foreign individuals, but this would imply a strong
327 reproductive bias between the two mother queens given their low frequency (6%). They could also
328 result from successive polygyny, but this would imply that in all colonies, queen replacement
329 occurred recently. Because the sampling was performed at the nest entrance, these workers are
330 more likely foraging workers from other colonies. It is important to note that *Cataglyphis* species are
331 non-territorial, and foreign workers are regularly collected during extensive sampling of excavated
332 colonies in the monogynous species *C. piliscapa* (Doums et al. 2013, 2018). We excluded these
333 workers to estimate the level of polyandry and relatedness because of their low frequency and
334 uncertain origin. The mean number of patriline observed was 2.44 in Salobralejo and 1.25 in
335 Castrillo (Table 1). Four out of the 13 colonies were monandrous (1 in Salobralejo and 3 in Castrillo).
336 Eleven patriline contained a single individual and were probably true patriline (not due to
337 genotyping errors) as they differed by three to nine loci from the other patriline found in the colony.
338 To determine whether the lower level of polyandry observed in Castrillo could be due to a smaller
339 sample size at this location, we resampled the Salobralejo colonies (except colony 19 that had a
340 smaller sample size than the Castrillo colonies) based on the Castrillo sample size. We estimated the
341 mean number of patriline from each resampling. From the 1000 simulations, we found that the
342 mean number of patriline in the resampled Salobralejo data (1.90 (95%CI: 1.5 – 2.5)) was less than

343 the full dataset (2.44). The observed number of patriline in Castrillo (1.25) falls outside the 95%
344 confidence interval of the resampled data suggesting that the lower level of polyandry may not
345 simply be due to a smaller sample size. Therefore, it is likely to reflect real biological differences
346 between the two populations.

347 In Castrillo and Salobralejo, the level of polyandry was less than *C. piliscapa* (Pearcy et al. 2004;
348 Doums et al. 2013). This could be due to lower genetic diversity. A decrease in genetic diversity
349 increases the probability that two fathers carry the same allele. However, we found that the genetic
350 diversity was similar to *C. piliscapa* and *C. cursor*. Further, the probability of not detecting two
351 unrelated fathers because they share the same allele is less than 0.0001% in both locations (Avila $p =$
352 0.0000004; Castrillo $p = 0.000003$). Alternatively, our smaller sample size (compared to *C. piliscapa*)
353 could have reduced our ability to detect patriline. As above, we resampled at random 15 colonies of
354 *C. piliscapa* from Argeles (Doums et al. 2013, 2018) with a known level of polyandry and frequency of
355 patriline reflecting the sample size of *C. cursor* from Spain. From the 1000 simulations, the mean
356 number of patriline estimated in *C. piliscapa* was 5.05 (95%CI: 2.75 – 4.75) based on the Castrillo
357 sample size and 3.86 (95% CI: 4.22 – 5.89) based on the Salobralejo sample size. The observed
358 number in Castrillo (1.25) and Salobralejo (2.44) were outside the 95% CI. This suggests that the
359 lower level of polyandry is unlikely to be due to a smaller sample size. The relatedness level was also
360 higher in Castrillo (0.68) compared to Salobralejo (0.47). The simulation of pairwise relatedness for
361 both locations using COANCESTRY showed that three out of eight colonies in Salobralejo and three
362 out of four colonies in Castrillo could belong to the $R = 0.75$ distribution (Fig. S2). None of the
363 colonies had a mean relatedness that could belong to the $R = 0$ distribution (Table 1).

364

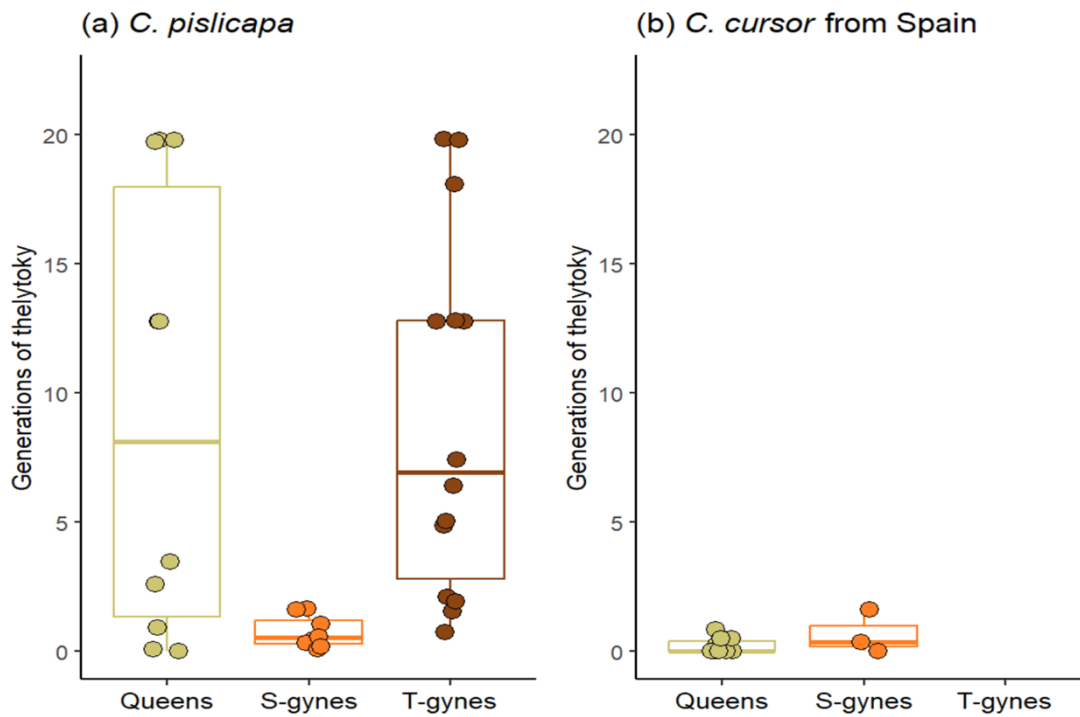
365 *Use of thelytoky*

366 From the population genetic analysis of workers, we found that there was no deviation from the HW
367 equilibrium. Clonal social hybridogenesis is associated with extremely high levels of heterozygosity in

368 workers due to queens mating with a distinct male genetic lineage that hybridise to produce workers
369 (Leniaud et al. 2012; Eyer et al. 2013). The absence of HW deviation suggests that these populations
370 do not use regularly clonal social hybridogenesis. Further, we found no evidence for the conditional
371 use of thelytoky to produce gynes. The nine gynes (from three colonies) were all sexually produced
372 (Table 2).

373 Using a maximum likelihood approach, we found minimal evidence for thelytokous reproduction
374 with a low estimated number of successive generations of thelytoky (median value < 0.001 with
375 maximum value of 1.62). This was based on the degree of homozygosity of the inferred mother
376 queens and gynes. The distribution of these estimates in *C. cursor* from Spain were significantly
377 different compared to gynes produced by thelytokous reproduction in *C. piliscapa* (Wilcoxon rank
378 test, $W = 3$, adjusted p -value with Bonferroni correction = 0.0003) and compared to mother queens
379 in *C. piliscapa* (Wilcoxon rank test, $W = 13$, p -adjusted = 0.027) (Fig. 4). However, we found similar
380 estimates to gynes produced by sexual reproduction in *C. piliscapa* (Wilcoxon rank test, $W = 25$, $p =$
381 0.12). Even though we cannot exclude the occasional use of thelytoky, we showed that thelytoky is
382 unlikely to be the main mode of queen production in *C. cursor* from Spain contrary to what is
383 observed in populations of *C. piliscapa*.

384



385

386 **Fig. 4** Box plot of the number of successive generations of thelytoky (SGT) required to explain the
 387 individual observed level of homozygosity. SGT was estimated by maximum likelihood for *C. piliscapa*
 388 (A) and *C. cursor* from Spain (B). Each dot represents the likelihood estimate of the SGT for an
 389 individual queen and a mean estimate by colony for gynes. The SGT estimates are provided for each
 390 mother queen, sexually produced gyne (S-gynes) and thelytokously produced gyne (T-gynes). We did
 391 not find T-gynes in *C. cursor* from Spain. See supplementary materials for further details on the
 392 maximum likelihood model.

393

394

395

396 Discussion

397 *A new putative species of the Cataglyphis group in Spain?*

398 The two populations of *C. cursor* sampled in a restricted geographical area in Spain (Fig. 1 and Fig. S1)
399 were genetically isolated (mtDNA and microsatellite markers) from the closely related species *C.*
400 *piliscapa* and *C. cursor*. The mtDNA genetic distances between these populations and those of *C.*
401 *piliscapa* and *C. cursor* were greater than those observed between populations of these two species
402 (Eyer et al. 2022). The two populations of *C. cursor* from Spain clustered together based on
403 microsatellite markers. In contrast, the populations of *C. cursor* and *C. piliscapa* overlap (Fig. 3).
404 However, more populations in this region (Fig. S1) should be analysed to confirm this pattern.
405 Bayesian phylogenetic inference (Fig. 1) placed the *C. cursor* populations from Spain within an early-
406 branching clade that is sister to the other *C. cursor* group species. Genomic data from a greater
407 number of species would improve our understanding of the evolutionary origins of these Spanish
408 populations. These populations could be ancient and may have persisted from the first colonisation
409 of Europe from the central Asian origin of the *cursor* group.

410 Morphological characteristics (the number of hairs on the scape and on the first segment of the
411 gaster) initially distinguished *C. piliscapa* from *C. cursor* (Agosti and Collingwood 1987). More
412 recently, this was confirmed by a quantitative assessment (Eyer et al. 2022). The workers of *C. cursor*
413 from Spain are completely hairless (C. Doums, pers. obs) like the Southern populations of *C. piliscapa*
414 (Eyer et al. 2022). A morphological assessment of workers, queens and males of these Spanish
415 populations is underway to determine whether these populations can be easily distinguished from *C.*
416 *piliscapa*. This morphological assessment could lead to the description of a new cryptic species of the
417 *C. cursor* group. This morphological study should be combined with cuticular hydrocarbons analyses.
418 Cuticular hydrocarbon analyses are an efficient way to identify cryptic ant species (Seppä et al. 2011;
419 Kather and Martin 2012; Villalta et al. 2018) and have been shown to differentiate populations of *C.*
420 *cursor* from Spain (Nowbahari et al. 1990). In ant species with a dependent colony foundation,
421 chemical recognition during the mating process is important because young queens remain at the
422 nest entrance and perform sexual calls to attract flying males from other colonies (Cronin et al.
423 2013). Recently, Eyer et al. (2022) showed that cuticular hydrocarbons can better identify differences

424 between *C. cursor* and *C. piliscapa* compared to male genitalia morphology. This supports the idea
425 that pre-mating isolation via chemical recognition may initially be more important than
426 morphological differences in species reproductive isolation.

427 Genetic differentiation between the two populations was high. This is also the case for *C. piliscapa*
428 and *C. cursor* (Eyer et al. 2022). However, more populations should be assessed to confirm this.
429 Within populations, we did not observe a pattern of isolation by distance with the microsatellite
430 markers. Given the geographical scale tested (approximately 200 m), the absence of isolation by
431 distance could be observed even under dependent colony foundation. Indeed, the population
432 genetic viscosity arising from restricted female dispersal (due to the dependent colony foundation)
433 could be reduced by the dispersal of flying males at this small spatial scale (Clémencet et al. 2005;
434 Hardy et al. 2008; Cronin et al. 2013). The low genetic diversity found for the mtDNA supports
435 dependent colony foundation as observed in *C. cursor* and *C. piliscapa*. Furthermore, the nine young
436 queens had a thorax morphologically like those of *C. cursor* and *C. piliscapa* queens (C. Doums, pers.
437 obs.) with a reduced mesothorax and had short wings suggesting that they were not able to fly and
438 found a colony alone. Indeed, contrary to voluminous thoraces of ant queens dispersing by flight,
439 flightless queens have a reduced mesothorax due to the loss of wing muscles (Peeters and Ito 2001;
440 Peeters and Aron 2017).

441

442 *Substantial variation in the breeding and reproductive system in the C. cursor group*

443 Three different reproductive systems can be found in the *Cataglyphis* genus: sexual reproduction
444 with arrhenotokous parthenogenesis (the classic system for species with haplodiploid sex
445 determination system) and conditional thelytoky with or without social hybridogenesis (Aron et al.
446 2016). The two populations studied here showed minimal evidence for thelytokous reproduction of
447 the queen. This confirms the lability of the reproductive system in this group with *C. italica* showing
448 conditional thelytoky with social hybridogenesis and *C. piliscapa* conditional thelytoky without social

449 hybridogenesis. We cannot assess the potential variability of the reproductive system in the Spain
450 region due to the limited number of populations studied. Interestingly, the minimal evidence of
451 thelytoky was associated with a lower level of polyandry in the two populations studied. This
452 supports the hypothesis that, in species using conditional thelytoky by the queen (without clonal
453 social hybridogenesis), thelytokous production of new queens should be observed under a high level
454 of polyandry associated with a worker-worker relatedness less than 0.5. This is because, from a
455 kinship perspective, workers collectively prefer rearing thelytokously produced queens than sexual
456 produced queens (Wenseleers and Van Oystaeyen 2011). This group provides a superb model system
457 to assess the relationship between polyandry and thelytoky.

458 The level of polyandry was lower in the two populations we studied than in *C. piliscapa* and
459 monoandry was even observed in four colonies (Table 1). These results are consistent with previous
460 work on the reversal from multiple to single mating that showed incomplete reversion (Sumner et al.
461 2004; Kronauer and Boomsma 2007; Aron et al. 2016). There can be multiple mechanisms underlying
462 the reduction in the level of polyandry. For example, it is associated with the evolution of a parasitic
463 lifestyle in *Acromyrmex* (Sumner et al. 2004) and with the evolution of polygyny in the Army ant
464 *Neivamyrmex carolinensis* (Kronauer and Boomsma 2007). In these systems, polyandry is no longer
465 required to maintain a genetic diversity in the worker force. Under these conditions, if polyandry is
466 costly, it may no longer be selected for (Kronauer and Boomsma 2007). These hypotheses are
467 unlikely in our system because our genetic analyses identified monogyny and *C. cursor* from Spain
468 does not have a parasitic lifestyle. In *Cataglyphis*, reversion to single mating has been reported three
469 times in the *C. altisquamis* and *C. emmae* groups (Leniaud et al. 2012; Amor and Ortega 2014; Aron
470 et al. 2016). In the *C. altisquamis* group, a decrease in polyandry was observed in a species with
471 clonal social hybridogenesis. The loss of genetic diversity among workers due to a reduction in queen
472 mating frequency may be compensated by the high level of heterozygosity in the workers (Aron et al.
473 2016). Clonal social hybridogenesis was not detected in our populations and is therefore unlikely to
474 have selected for lower polyandry. In the *C. emmae* group, polyandry is reduced in two sexually

475 reproducing monogynous species characterised by very small colony sizes (a few dozen individuals)
476 (Aron et al. 2016). In our study, colonies were only partially excavated, therefore, we could not
477 assess colony size. However, colonies are constituted by hundreds and thousands of workers in the
478 related *Cataglyphis* species and most species of the genus. Consequently, this hypothesis is also
479 unlikely to be supported. Interestingly, in all these above hypotheses, a decrease in polyandry is
480 observed when selection for the maintenance of a highly diverse genetic worker force is relaxed
481 (Crozier and Fjerdingstad 2001; Strassmann 2001).

482 We present a novel hypothesis that has not been proposed in previous studies: the benefits of
483 multiple mating are reduced when most mating occurs with genetically similar males. As a result,
484 multiple mating fails to increase genetic diversity among workers to compensate the costs.
485 Therefore, lower levels of polyandry would be expected in isolated populations. In birds, the level of
486 extra-pair paternity (another way to achieve multiple mating) was found to be lower in islands
487 compared to mainland populations in agreement with this hypothesis (Petrie et al. 1998). This
488 hypothesis is relevant to our study because of the limited distribution area and geographical isolation
489 of the populations. The level of polyandry can also directly affect population diversity especially by
490 affecting N_e (Karl 2008). This can lead to a feedback loop. The predicted positive correlation between
491 polyandry and the level of heterozygosity has been observed across multiple taxa (Taylor et al. 2014).
492 However, this hypothesis is unlikely to explain the lower number of mates in *C. cursor* from Spain
493 because the genetic diversity observed was not lower than in populations of *C. piliscapa* or *C. cursor*,
494 and we did not detect inbreeding. Nevertheless, the relationship between genetic diversity and
495 polyandry would merit further investigation in ants. Finally, ecological factors affecting the
496 availability of mating partners, parasitism pressures, foraging strategies, and the diploid male load
497 due to haplo-diploid sex determination system could also affect the costs and benefits of polyandry
498 and need to be assessed.

499 The present study has expanded our knowledge of an intriguing group from the genus *Cataglyphis*.
500 This work provides the foundation for investigating the interplay between the evolution of polyandry
501 and thelytoky in this group. The two populations in central Spain are genetically monogynous
502 without queen inbreeding. This suggests that thelytoky is absent or rare. This was also supported by
503 the nine gynes we analysed. These populations also exhibit low levels of polyandry. Ongoing
504 morphological and cuticular hydrocarbon research will allow us to describe in detail a potentially new
505 sister species. Our results demand further analyses to assess the relationship between the evolution
506 of polyandry and thelytoky and to understand the phylogenetic origin of this potential species.

507

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512

513 **Statements and Declarations**

514 CD, IV and RB contributed to the study conception and design. Data collection was performed by IV
515 and RB, molecular work was performed by PCB and data analysis was performed by CD with
516 contribution of TL. The first draft of the manuscript was written by CD and all authors commented on
517 previous versions of the manuscript. All authors read and approved the final manuscript.

518 The authors declare that they have no conflict of interest.

519 mtDNA new haplotype sequences are available in GeneBank with the accession numbers from
520 OR529503 to OR529507 and microsatellite data are available at
521 <https://doi.org/10.5281/zenodo.7977735>.

522 Ant species are excluded from legislation on animal use in research, but care was taken to minimize
523 stress and quickly kill the ant during field collection.

524 **Table 1:** Sociogenetic organisation of *C. cursor* from Spain. N.offspring = number of female offspring
525 genotyped (w if workers and g if gynes). Gynes were found in only three colonies. The number of
526 foreign offspring detected is given in parentheses. All foreign offspring had at least three loci with no
527 common alleles with the putative mother queen of the other females. These alien offspring were not
528 considered when estimating polyandry parameters. All genotyped gynes were sexually produced and
529 included when calculating polyandry estimators. The number of patriline (Np) and effective mating
530 (ke) are given. The Queller & Goodnight estimate of relatedness among workers within each colony is
531 also given as Rw , with its variance in parentheses. Rw fall within the hypothesis of $R = 0.5$ for all
532 colonies (Fig. S2), for the six values in bold, the Rw was also consistent with the $R = 0.75$ hypothesis.

Site	Colony	N.offspring	Np	Ke	Rw
Salobralejo	4	12w	1	1	0.66 (0.01)
Salobralejo	7	11w	2	1.2	0.65 (0.03)
Salobralejo	8	9w+3g (1w)	2	1.7	0.35 (0.11)
Salobralejo	9	5w+4g (1w)	4	3.6	0.43 (0.06)
Salobralejo	11	12w (1w)	3	2.0	0.31 (0.10)
Salobralejo	13	12w (1w)	2	1.2	0.52 (0.05)
Salobralejo	15	11w (1w)	3	2.5	0.40 (0.07)
Salobralejo	19	4w+2g	2	1.4	0.51 (0.10)
Salobralejo	27	10w (1w)	3	2.2	0.42 (0.12)
Mean Salobralejo			2.44	1.87	0.47
Castrillo	31	7w (1w)	2	1.4	0.42 (0.08)
Castrillo	46	7w	1	1	0.75 (0.01)
Castrillo	54	8w	1	1	0.73 (0.01)
Castrillo	59	6w	1	1	0.68 (0.01)
Mean Castrillo			1.25	1.1	0.64

533

534 **Table 2:** Genotypes of the gynes found in three colonies of Salobralejo. Colony number (col.) and
535 individual codes (code) are provided for each gyne as well as the informative loci. We considered a
536 locus to be informative when comparing a pair of gynes from the same colony. Three alleles were
537 observed thereby excluding the two gynes as clonal sisters (see Doums et al. 2013 for more details).
538 We emphasise in bold the alleles that allowed us to identify a pair of sexually produced gynes. Note
539 this criterion is conservative and is not powerful at discriminating full-sister gynes from thelytokously
540 produced gynes (as it does not take into account LOH). In spite of this, all pairs of gynes did not fulfil
541 this criterion based on a number of discriminating loci varying from 1 (i.e. pair 8G1 and 8G3) and 5
542 (i.e. pair 8G1 and 8G2).

543

Col.	Code	L89	L61	L99	L100	L10878	L2794	L3653	L3993
8	8G1	114/118	213 /235	104/106	183/185	296/300	269/269	242/248	363/366
8	8G2	114/ 120	229/235	104/ 108	183/183	288 /300	265/269	242/248	366/ 372
8	8G3	114/118	229/235	104/106	183/183	296/300	265/269	242/248	363/366
9	9G1	114/118	215/229	104/106	183/183	296/302	259/269	248/248	361/ 363
9	9G2	114/118	215/229	104/ 108	183/185	296/302	259/ 265	248/248	361/372
9	9G3	114/ 120	213 /229	104/106	185/185	280 /296	253 /269	248/256	361/372
9	9G4	114/118	215/229	104/ 108	183/185	296/302	259/265	248/248	361/372
19	19G1	116/118	213/213	104/106	185/187	312/312	244/253	242/248	357/361
19	19G2	118/118	213/215	104/106	183 /185	280/314	244/ 259	248/ 254	361/361

544

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