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Integrative characterization of genetic and phenotypic differentiation in an ant species complex with strong hierarchical population structure and low dispersal abilities

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Low dispersal, occurrence of asexual reproduction and geographic discontinuity increase genetic differentiation between populations, which ultimately can lead to speciation. In this work, we used a multidisciplinary framework to characterize the genetic and phenotypic differentiation between and within two cryptic ant species with restricted dispersal, *Cataglyphis cursor* and *C. piliscapa* and used behavioral experiments to test for reproductive isolation. Their distribution is segregated by the Rhône River and they have been traditionally distinguished only by hair numbers, although a statistical assessment is still lacking. We found strong genetic (microsatellites, nuclear and mitochondrial sequences), morphological (number of hairs, tibia length, male genitalia) and chemical (cuticular hydrocarbons) differentiation not only between species but also among localities within species. However, inter-specific differentiation was slightly higher than intra-specific differentiation for most markers. Overall, this pattern could either reflect reproductive isolation or could result from a longer period of geographic isolation between species than among localities within species without necessarily involving reproductive isolation. Interestingly, our behavioral experiments showed an absence of mating between species associated to a higher aggressiveness of workers towards heterospecific males. This suggests that sexual selection may, at least partially, fuel reproductive isolation. We also showed that cuticular hydrocarbons, mtDNA sequences and number of hairs provide reliable criteria allowing species discrimination. Overall, this species complex offers a case study to further investigate varying stages of a speciation continuum by estimating reproductive isolation between pairs of localities varying by their level of genetic differentiation.

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INTRODUCTION

Speciation is usually a long evolutionary process through which a population splits into different evolving lineages that ultimately give rise to sister species (De Queiroz 2007). This segregation is usually triggered by selection and/or geographic isolation which, over time, leads to the accumulation of barriers to gene flow resulting in reproductive isolation (Mayr 1942; Endler 1973; Coyne and Orr 2004; Wang et al. 2013; Sexton et al. 2014; Richardson et al. 2014). Before reaching complete isolation, divergent populations can harbor various levels of reproductive isolation that can be positioned on a speciation continuum (Stankowski and Ravinet 2021b) and are often coupled with different levels of phenotypic and genetic differentiation (De Queiroz 2007; Hendry et al. 2009). Defining species boundaries along a speciation continuum is therefore complex and not always possible as it strongly relies on the criteria used to distinguish species. Integrative approaches relying on genetic and phenotypic differentiation associated to mating experiments directly assessing reproductive isolation should help distinguishing within-species structure from species boundaries (Schlick-Steiner et al. 2010).

By affecting gene flow, distinct life history traits, such as dispersal ability and reproductive systems, may profoundly affect the speciation process (e.g., Bolnick and Otto 2013; Servedio 2016; Slatkin 1993). Low dispersal hence favors speciation events over restricted spatial scale (Kisel and Barraclough 2010) and enhances the establishment and persistence of within-species lineages that can ultimately lead to new species (Dynesius and Jansson 2014). Species with weak dispersal abilities might therefore be particularly prone to the evolution of reproductive isolation over small geographic scale (Pante et al. 2015). Similarly, the transition to asexual reproduction enhances speciation when asexual lineages become completely isolated from the sexual ancestors (Neumann et al. 2011; Gebiola et al. 2012; Elias-Costa et al. 2019). Even when facultative, asexual reproduction might strongly impede gene flow. Species combining these two characteristics, limited dispersal and asexual reproduction, usually exhibit strong

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population structure. The use of multidisciplinary approaches is therefore particularly relevant to understand in which stage of the speciation continuum they are, as genetic data alone might not convey enough information.

In this study, we investigate genetic and phenotypic differentiation between and within two sibling species of the thermophilic ant genus *Cataglyphis*, known to have low dispersal abilities, and further coupled these analyses with behavioral experiments. The species status of *C. cursor* and *C. piliscapa* have long been debated (note that most of the publications based on localities of *C. piliscapa* wrongly used the species name *C. cursor*). Their identification is primarily based on the number of hairs on the first tergite of the gaster (Bondroit 1918; Agosti and Collingwood 1987) and on cuticular hydrocarbon analyses (Nowbahari et al. 1990), but a robust quantitative assessment is still lacking. Interestingly, these two allopatric species are separated by the Rhône River in the Southern France, with *C. cursor* occurring on the East side and *C. piliscap*a on the West side (Nowbahari et al. 1990). In both species, female dispersal abilities are particularly weak, consistently with the low number of colonies produced each year and the widespread adoption of the fission process (Lenoir et al. 1988; Cronin et al. 2013). During colony fission, young queens of both species leave the natal nest on foot with nestmate workers to found few new colonies nearby (four new nests at 7 m of distance on average) (Lenoir et al. 1988; Chéron et al. 2011). Colonies of both species are headed by a single queen mated with several males (Lenoir et al. 1988; Pearcy et al. 2004). Four main factors limit male gene flow in *C. piliscapa*: i) males are not good flyers and their effective dispersal distance was estimated to be less than 250 m (Hardy et al. 2008); ii) male gene flow is restricted to the pre-mating process since queen dispersal occurring after mating is limited to the worker's walking distance; iii) workers aggress males during mating, acting as a potential filtering process and indirect source of sexual selection (Helft et al. 2015). By aggressing differentially males from different localities of origins, workers might therefore also play a role in limiting the mating efficiency of immigrant males. iv) Queens mate with males from different colonies to produce workers but use thelytokous parthenogenesis (originating daughters by asexual reproduction) to produce part, if not all, of the new queens (Doums et al. 2013; Pearcy et al. 2004). The remaining fraction of the new queens is produced by sexual reproduction. Queens also reproduce through arrhenotokous parthenogenesis (originating males by asexual reproduction) to produce new haploid males, as it is classically the case in Hymenoptera (Trivers and Hare 1976). This unusual reproductive system reduces male gene flow and limits genetic mixing in reproductive individuals, as most of the new queens and all males are asexually produced. Males contribute to gene flow through the small fraction of sexually produced queens (Fig. 1A). Occasionally, males can also contribute to gene flow in orphaned colonies, as workers can asexually produce new males and queens in the absence of the mother queen (Fig. 1A). These reproductive characteristics result in a strong genetic structure at fine spatial scale (from 100 m up to 120 km; Clémencet et al. 2005; Hardy et al. 2008), and may therefore favor evolution of reproductive isolation over small geographical scale. It is not yet clear whether *C. cursor* shares the same reproductive system and its population genetic structure is not yet known.

Here, we used an integrative approach to investigate the level of genetic and phenotypic differentiation between localities both within and between the two defined species *C. piliscapa* and *C. cursor*. We aimed to detect the highest possible within-species differentiation by sampling localities either separated by a geographic barrier (the Durance River for *C. cursor*) or at the extreme ranges of the geographic distribution (for *C. piliscapa)*. To this end, we compared the level of phenotypic and genetic differentiations within vs. between species by: i) considering three types of genetic markers (nuclear and mitochondrial sequences, and microsatellite markers); ii) measuring morphological traits (number of hairs, tibia length and morphology of male genitalia); and iii) performing a chemical analysis of the cuticular hydrocarbons. Finally, we further investigated mechanisms of reproductive isolation performing behavioral experiments to test for the mating success of conspecific and heterospecific males as well as the aggressive behavior of workers towards them.

MATERIALS AND METHODS

Colony sampling

Between 2015 and 2017, workers from 369 distinct colonies covering 26 localities were sampled in Southern France and Northern Spain (Fig. 1B; Table S1). For *C. cursor*, we sampled 15 localities on each side of the Durance River (213 workers), whereas for *C. piliscapa*, we sampled 11 localities at the two extreme ranges of its geographic distribution (156 workers). For all subsequent analyses, individuals were assigned to the species according to their geographic position, but we did not use any other a priori to assign individuals to a given species. Given the presence of a putative barrier to gene flow for *C. cursor* (the Durance River) and the sampling gap between the Northern and Southern distribution of *C. piliscapa* (Fig. 1B), we refer for all the following analyses to four sampled regions, two for each species (Fig. 1B). For a subset of these colonies ($n = 241$), one individual per colony was cooled down in an icebox immediately upon collection and frozen at the end of the day for later use for chemical analyses (Table S1). The remaining samples were stored in 95% ethanol (5%TE; Tris 10 mM: EDTA 1 mM) until DNA extraction. One worker per colony ($n = 369$) was genotyped at 13 microsatellite markers. Due to expected low within-locality polymorphism (Clémencet et al. 2005), we only sequenced two workers per locality ($n = 52$ individuals) for the COI mitochondrial marker and 12 EPIC nuclear genes. We chose individuals from the most distant colonies in order to capture most of the total genetic variability (Clémencet et al. 2005). All genotyped workers were measured for the number of hairs on the first tergite of the gaster and the length of the tibia ($n = 369$). An additional dataset of 92 males was used to assess morphological differences in male genitalia. These males were obtained from 21 colonies sampled in seven localities (four localities of the genetic sampling and three news ones) distributed within the four regions (Fig. 1B, Table S1).

Genetic analysis

Total genomic DNA was extracted from half of the head using the extraction kit Qiagen (DNA Easy blood and tissue kit). One mitochondrial locus (Cytochrome Oxidase I, COI) was amplified following the protocol of Clémencet et al. (2005). Twelve Exon-Primed-Intron-Crossing nuclear loci (EPIC) designed by Ströher et al. (2013) were sequenced (see loci characteristics and sequencing conditions in Table S2). Two individuals from *C. italica* (from Bari, Italia) were also sequenced and used as outgroup. PCR products of nuclear and mitochondrial loci were sent to GENOSCREEN (Lille, France) for Sanger sequencing. Base calling and sequence alignment were performed using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). Phase reconstruction in nuclear loci is detailed in Table S2. All DNA sequences were deposited in GenBank database (accession numbers in Table S2). In addition, thirteen microsatellite loci were amplified for genetic analyses (see characteristics and PCR conditions in Table S3).

All analyses (genetic, morphological, and chemical) were conducted on R v.3.2.2 (R Core Team 2016) except when otherwise stated.

EPIC loci. The Haploweb approach was used to delimit genetic clusters using phased haplotypes (alleles) of EPIC loci that co-occur within heterozygous individuals (Flot et al. 2010). Haploweb uses haplotype networks to identify connections between haplotypes co-occurring in heterozygous individuals to visualize and delineate single-locus 'fields for recombination' (FFRs). Haplotype networks were built with NETWORK v.10.2 using the median-joining method (Bandelt et al. 1999). The congruence of FFRs obtained from several independent loci is used to estimate clustering support. The congruence between nuclear markers is reported on a haploweb matrix. This matrix represents, for each pair of individuals, a score equal to the number of EPIC markers supporting the clustering of the two individuals (i.e., for which the two individuals belong to the same FFR). The low genetic diversity observed for the nuclear sequences did not allow inferring summary statistics and testing the correlation between genetic and geographical distances.

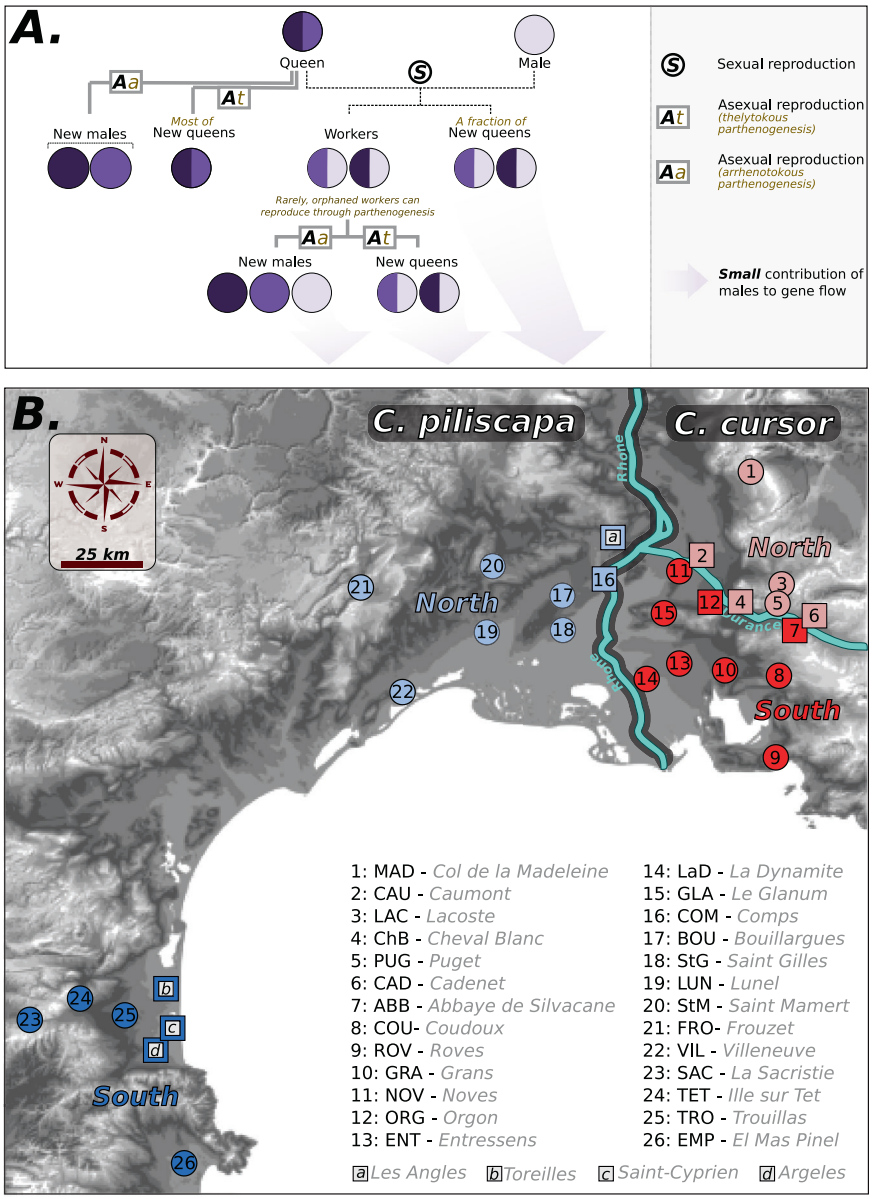

Fig. 1 Schematic representation of the mating system observed in *C. piliscapa* and sampling localities of *C. cursor* and *C. piliscapa*. **A** Schematic representation of the mating system observed in *C. piliscapa*. Individuals are colored according to their genetic background. Faint arrows represent the small contribution of males to gene flow. **B** Sampling localities of *C. cursor* and *C. piliscapa*. Each locality is colored according to its species and region of origin (red for *C. cursor* South region, pink for *C. cursor* North region, dark blue for *C. piliscapa* South region and light blue for *C. piliscapa* North region). Samples used for genetic, morphological and chemical analysis are coded by a number in a circle whereas the four localities used for genitalia measurements and/or behavioral experiments are coded by a letter in a square. Detailed sample size for each analysis and each locality can be found in Table S1.

mtDNA. Bayesian Inference (BI) was used to reconstruct phylogenetic relationships among haplotypes using the program MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). The two samples of the sister species *C. italica* from Bari were used as outgroup. Tree topology and nodes support were estimated using a Metropolis-coupled Markov chain Monte Carlo sampling approach, with two runs implementing four chains (three hot, one cold) running for 1×10^7 generations, saving one tree every 100 generations. Convergence was checked by examining the standard deviation of the split frequencies between the two runs (<0.01) and the Potential Scale Reduction Factor diagnostic. The first 25,000 trees of each run were discarded as burn-in, and a majority-rule consensus tree was generated from the 75,000 remaining trees.

The genetic distance between localities was computed as the average proportion of nucleotide differences (p-distance). Visualization of the pairwise distance matrix was obtained by means of a non-parametric multidimensional scaling plot (NMDS) as implemented in the MASS library. Correlation between geographic and genetic distances was assessed by a Mantel test (Mantel 1967) computed with the VEGAN library. We further tested the influence of the Rhône River on genetic differentiation using a partial Mantel test. To this end, we built a binary pairwise matrix with values of 0 corresponding to pairs of localities separated the river (belonging to different putative species) and 1 for pairs of localities on the same side of the river (belonging to the same species).

Microsatellite data. We tested for linkage disequilibrium between loci and for Hardy-Weinberg equilibrium within each locality using exact tests within the Genepop Package (Rousset 2008). Allelic richness was estimated using the rarefaction method (it standardizes to the smallest number of individuals sampled across all combinations of locus and locality multiplied by the ploidy level of the species) implemented in the package *PopGenReport*, allowing to control for different sample size (El Mousadik and Petit 1996). We used a mixed effect model with locus as random factor

to test for a difference in allelic richness and gene diversity between the two species (considered as fixed factor in the model) using the package *nlme*. Pairwise G_{ST} (Hedrick 2005), which corrects for the F_{ST} maximal value given the observed within locality diversity, was estimated with the *PopGenReport* package. Similarly to mtDNA, G_{ST} distance matrix was visualized by an NMDS and the effects of the Rhône River and geographical distance on G_{ST} were tested using the same partial Mantel test. We run the Bayesian clustering approach implemented in STRUCTURE v.2.3 (Pritchard et al. 2000; Falush et al. 2007), varying K (number of clusters) from 1 to 26 (i.e., number of localities). We run the algorithm 10 times for each K with a combination of an admixture and a correlated-allele frequencies model. Each run included a 5×10^4 burn-in period followed by 1×10^5 iterations of the MCMC. Data convergence was assessed by analyzing Alpha equilibrium across iterations. The most likely number of genetic clusters was evaluated using the ΔK statistics (Evanno et al. 2005) and the log-likelihood value generated by Structure Harvester v.0.6.8 (Earl and VonHoldt 2012).

Chemical analyses of cuticular hydrocarbons

For each colony sampled in 2015 (the South region of *C. piliscapa* distribution was missing), chemical extractions were carried out a few weeks after sampling, with each worker individually retrieved from the freezer immediately before its chemical extraction. After removal of the gaster, the individual's body was immersed in 200 µl of pentane for 5 min to extract the cuticular hydrocarbons. Samples were analyzed by gas chromatography-mass spectrometry following the protocol described in Table S4. Peak areas were integrated with MSD ChemStation software v.E.02.01.1177 (Agilent technologies). Chemical compounds were identified by their retention times and fragmentation patterns, and were confirmed through comparisons with previous results on *C. piliscapa* (Monnin et al. 2018; Table S4).

To assess the chemical differentiation between the two species and between the two regions within *C. cursor*, we used a Random Forest analysis (RF) with the R package *randomForest* (Liaw and Wiener 2002). RF is a tree-based machine learning tool increasingly used in chemical studies, including the chemical ecology of social insects (i.e., Mitra et al. 2014; Monnin et al. 2018) as it deals well with multicollinearity, assumes no specific distribution of the variables and does not require transformation of constrained data (such as chemical compounds) (Ranganathan and Borges 2010). We used the relative abundances of peaks (i.e., the proportion each peak represented relative to the sum of all peaks) computed from the raw dataset, including all the zero values. We performed RF using 10,000 trees to determine the importance of each peak in classifying the individuals into the two species. We used the default values for the number of input variables randomly selected to build each node of the tree, and for the number of observations not used for building the current tree (i.e., the out of bag sample—OOB). This OOB sample (33% of individuals) was used to generate the confusion matrix and the OOB error rate (i.e., rate of unsuccessful assignment of the OOB samples to the correct species). A low OOB error rate indicates a high ability of the indicator variables to predict the species of origin of the individual. The pairwise proximity matrix between individuals estimated by the RF was visualized using a metric multidimensional scaling plot and was used to perform a Mantel test for testing the correlation between chemical and genetic distances (using G_{ST}). The individual chemical distances were averaged to obtain mean chemical distances between localities before performing the Mantel test.

Morphometric analyses of workers

The left foreleg of each genotyped worker ($n = 369$) was detached from the body, glued on a double-faced tape, and the tibia was measured to the nearest 0.01 mm using a stereomicroscope (Leica Microsystems, Wetzlar, Germany) and the software ImageJ v.1.8 (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij/). In addition, we counted the number of hairs on the first tergite of the gaster. This trait is the main criterion used to discriminate *C. cursor* and *C. piliscapa*, the former having more than five hairs whereas the latter has less than five (Agosti and Collingwood 1987).

We first used the R library pdfCluster (Azzalini and Menardi 2014) to cluster individuals based on the two morphological variables. This package performed clustering based on nonparametric density estimation. The first phase consists in determining space regions (cluster cores) containing objects with low dissimilarities and separated from each other by valley of high dissimilarities. The second phase consists in assigning the data points still unlabeled (those not falling into a determined cluster core) among the cluster cores. We used the default arguments proposed by the function pdfCluster. We performed the clustering based on both variables.

Second, we investigated the differences between the two species using a mixed effect model with the package *nlme* (Pinheiro et al. 2021), considering the species as a fixed effect whereas localities were considered as a random effect. We allowed heterogeneous variance between species for the number of hairs using the weight function (*varIdent*). We checked visually that residuals follow model assumptions for both dependent variables. The fixed effect was tested by comparing the model fitted with the maximum likelihood method with and without the fixed factor using a log-likelihood ratio test.

Geometric morphometrics of male genitalia

The 92 male genitalia were dissected under a binocular microscope after a 4-h bath in KOH 10%, and only the left side of the genitalia was used for measurement. Four sclerified elements of the genitalia were separated (sternum IX, paramere, volsellae and penisvalvae; Boudinot 2013) and digitalized using a Leica M165C binocular microscope connected to a Leica DFC425 camera. Anatomical landmarks and sliding semi-landmarks were defined for each element using the software tpsDig2 (Gunz and Mitteroecker 2013; Rohlf 2015). Five anatomical landmarks and 18 sliding semi-landmarks were placed on each sternum IX (paramere: 6 landmarks/44 semi-landmarks; volsella: 5 landmarks/42 semi-landmarks and penisvalvae: 4 landmarks/35 semi-landmarks). These landmarks were used to estimate the shape of each element for every individual. The sliding phase was performed by minimizing the bending energy between each specimen and the consensus (Gunz and Mitteroecker 2013). The coordinates were then superimposed using a Generalized Procrustes Analysis (Rohlf and Slice 1990) with the R library *geomorph* v.3.0.6 (Adams et al. 2018). This analysis removes the effect of size, location, and orientation. The centroid size (the square root of the sum of the squares of the distance from each point to the centroid of the set of landmarks), a measure of size, was also taken into account for the following analyses to control shape variation for size.

For each element of the male genitalia, variation in conformation between individuals was analyzed with a Principal Components Analysis (PCA). The first components accounting for at least 90% of the PCA total variation were used as new variables to define the shape of each element (shape variables) (Baylac and Frieß 2005). This procedure reduces the dimensionality of the data set and works on independent variables (Baylac and Frieß 2005). In order to reach 90% of the variation, five components were used as shape variables for sternum IX, eight for paramere, nine for volsellae, and seven for penisvalvae. Multivariate analyses of variance (MANOVA) were performed to test for statistical difference in shape for each genitalia element. Three MANOVAs were performed. The first one was used to test for difference between species and the other two were used to test for difference between regions within each species. We used centroid size, the square root of the sum of the variances of the landmarks around the centroid in *x* and *y* directions to control for size in our analysis, as in the absence of allometry the centroid size is supposed to be uncorrelated with any shape variable (Bookstein 1986; Jungers et al. 1995). Shape variations between species and regions were visualized using a canonical variate analysis (CVA) for each of the four elements.

Behavioral experiments

We used seven colonies from five localities that were collected either during the sampling described in this paper or in a previous sampling (Table S1). Colonies hibernated in the laboratory at about 14 °C, and sexuals were produced once colonies were removed from hibernation. Males were removed from their colonies twice a week to prevent intra-nest mating and ensure that young queens remained virgins (gynes). Gynes were marked every three to four days to record their age. A total of 11 gynes (six *C. piliscapa* and five for *C. cursor*) and 22 males (11 for each species) were used (Table S5).

Each behavioral experiment was divided into three successive trials of 10 min separated by 15 min of resting time between two trials and was conducted with the same individuals. Each behavioral experiment took place in a mating box (10.5*9.5 cm) under natural light complemented with a heating lamp to raise temperature to ~35 °C. This mimics mating conditions in the field, which occurs in spring during the hottest hours of the days. In *Trial 1*, we placed one gyne and two males (one per species) in the mating box, to test for unimpeded mate choice by the gyne. After having removed the males, we added 15 workers originating from the same colony of the gyne (i.e., nestmates) in the mating box and let them

15 min for acclimation. *Trial 2* began when the two previous males were placed again in the mating box for 10 min, to test for the behaviors of workers towards males. Workers may differentially aggress the conspecific and heterospecific males and thus affect their mating success, as previously suggested within *C. piliscapa* (Helft et al. 2015). After having removed the males, we let again the gyne and workers rest for 15 min. *Trials 3* began when we added two foreign workers, each one being a sister of the males previously tested in *Trials 1* and *2* (i.e., originating from the same colonies). The idea was to test whether workers behave more aggressively towards heterospecific than conspecific workers, and to test whether this effect was stronger when targeting males (*Trial 2*) or workers (*Trial 3*). In all trials, we paid attention to select males that are dark pigmented, active, moving around, and excited with their wings open to ensure their readiness to mate. Note that workers of *C. piliscapa* and *C. cursor* are not territorial and are generally peaceful with workers from colonies of the same locality, which can easily be adopted in natural colonies (Nowbahari and Lenoir 1989) and are found at low frequency in field colonies (Doums et al. 2018). We performed 11 behavioral experiments, five with a *C. cursor* gyne (all originating from a single colony) and six with a *C. piliscapa* gyne (five gynes from one colony and one gyne from another one) (see Table S5). The conspecific foreign male and workers came from a different locality than the gyne. They were from the same region in five experiments (with *C. piliscapa* gynes) and from a different region in six experiments (five experiments with *C. cursor* gynes and one experiment with *C. piliscapa* gyne) (Table S5). Unfortunately, the low number of sexuals produced did not allow us to robustly test for the occurrence of indirect sexual selection within each species, calling for a cautious interpretation of the results.

Each trial of 10 min was videotaped with a Sony Handycam HD and the behaviors were recorded using the free software BORIS v.7.4.10 (Friard and Gamba 2016). Before introduction into the mating box, the two males and two foreign workers were marked with a color dot on the thorax using UniPaint Marker. In *Trials 1* and *2*, we recorded the number of mating attempts (as described in Helft et al. 2015) and the number and duration of successful matings (male and gyne locked into mating). In *Trials 2* and *3*, we recorded the duration of biting of workers towards foreign males or workers. Workers sometimes hold biting for a prolonged period of time so that the number of biting alone does not appropriately reflect the aggression intensity.

Using data from *Trials 1* and *2*, in which males were present, we tested whether the number of mating attempts differed as a function of the origin of males (conspecific versus heterospecific), of the presence/absence of workers, and of the gyne species (*C. cursor* or *C. pilliscapa*) using a mixed effect model with the behavioral experiment as a random factor. Each main fixed effect was tested by comparing the full model with a reduced model without the tested main effect using a log likelihood ratio test. Homoscedasticity and normality of residuals were checked visually. Using data from *Trials 2* and *3*, in which workers that are sisters of the gyne were present, we tested whether the duration of aggressive behaviors differed as a function of the origin of individuals (conspecific versus heterospecific), of the type of foreign individuals (males or workers) and the gyne species (*C. cursor* or *C. pilliscapa*) using the same model than above. In this model, we included the interaction between the type of individuals introduced (males or workers) and their origin, as the behavior of worker sisters of the gyne towards introduced individuals is expected to differ according to their type. When the interaction was significant, mean comparisons were performed using the package *emmeans*.

RESULTS

Genetic analysis

Nuclear sequences. Nine out of the twelve nuclear EPIC markers were successfully sequenced for all individuals analyzed ($n = 52$, Table S2). After reconstructing the phase for the nine markers, we found an average of 8.8 haplotypes and 9.4 SNPs per marker (Table S2). We found a single SNP (EPIC 1503) discriminating the two species, (i.e., without shared alleles between the two). Haploweb uncovered three main genetic clusters (Fig. 2): one cluster grouping all *C. cursor* localities, and two others grouping all *C. piliscapa* localities (the haploweb for each EPIC marker are given in Fig. S1). The outgroup *C. italica* was well differentiated. The two distinct clusters within *C. piliscapa* (dark and light blue color, Fig. 2B) match the geographical regions except for the localities of VIL and FRO. However, although the Northern localities of *C. piliscapa* form a homogenous cluster, the result is less evident for the South region of this species. Within *C. cursor* (dark and light red color, Fig. 2B), two clusters could also be distinguished (even though less clearly) and differentiate the two sides of the Durance River, except for the localities CAD and ROV.

MtDNA. The mitochondrial COI marker was successfully sequenced for all individuals analyzed ($n = 52$). We found 23 unique haplotypes defined by 93 SNPs, representing 16% of the total sequence. In 21 out of the 26 localities, the two individuals sequenced shared the same haplotype. Five SNPs were fixed for a different allele in each species, potentially representing markers for species identification. The phylogenetic tree (Fig. 2A) identifies two main clusters corresponding to the two species. The mean p-distance between regions of *C. piliscapa* was 0.061 and of 0.029 between regions of *C. cursor*, while the p-distance between species was 0.064. This was reflected in the pattern shown by the NMDS plot (Fig. 3A), as it separated only three main clusters that were more or less genetically equidistant. This analysis identified the two clusters previously uncovered within *C. piliscapa* (except for localities FRO and VIL). Within *C. cursor*, the two sides of the Durance River were only moderately discriminated, with discrepancies for the localities CAD and ROV. We found a significant positive association between the mean p-distance and geographical distances after controlling for the presence of the Rhône River with the partial Mantel test ($r = 0.40$; $P = 0.001$, Fig. 4A). Similarly, the presence of the Rhône River separating pairs of localities also significantly increased genetic differentiation after controlling for the geographical distances (partial mantel test, $r = 0.61$; $P = 0.001$), showing that for a given geographical distance, localities from the two species were more genetically differentiated than within species. This was always true for *C. cursor* but not for *C. piliscapa* (Fig. 4A), where some pairs of localities showed the same level of genetic distance than pairs of localities from the two species (the matrix of pairwise genetic distances is provided in Table S6). These pairs of localities were those including one locality from each of the two genetic clusters identified by the NMDS plot within *C. piliscapa* (i.e., separating FRO, VIL, EMP, TET, SAC, and TRO from the rest of the localities of this species).

Microsatellite data. We did not detect linkage disequilibrium between microsatellite loci (Table S7) and all except one HW equilibrium tests were not significant after Bonferroni's correction (Table S8). All 13 microsatellite loci used in this study were polymorphic with the allelic richness per locality and locus ranging from 1 to 7.37 (mean ± SD = 3.65 ± 1.49) and gene diversity from 0 to 0.95 (mean ± SD = 0.61 ± 0.24) (Table S9 and S10). Interestingly, C. *piliscapa* presented a significantly higher allelic richness (4.22 ± 0.93) than *C. cursor* (3.23 ± 0.32) for all but one locus (mixed effect model, L-ratio = 55.23, $P < 0.0001$, Table S9). Gene diversity showed a similar pattern (*C. piliscapa*: 0.68 ± 0.12; *C. cursor*: 0.56 ± 0.06; mixed effect model: L-ratio = 28.18, $P < 0.0001$, Table S10).

Highly significant population differentiation was found among all but three comparisons (these three nonsignificant comparisons were found between neighboring localities of *C. cursor* in the South region, Table S11). The global Hedrick's G_{ST} was high (0.73 ± 0.20) (pairwise values of G_{ST} and F_{ST} are given Table S11). The genetic differentiation was always high between species (mean ± sd, $G_{ST} = 0.81 \pm 0.07$) as well as between regions for both species (mean ± sd *C. cursor*: $G_{ST} = 0.81 \pm 0.09$; *C. piliscapa* $G_{ST} = 0.80 \pm 0.08$) whereas it was more variable, with a lower mean when comparing localities within region for both species (mean ± sd *C. cursor*: $G_{ST} = 0.43 \pm 0.24$; *C. piliscapa* $G_{ST} = 0.59 \pm 0.27$) (Table S11). Localities from the same geographic regions clustered together (with two exceptions, FRO and EMP, see Fig. 3B) but the four clusters

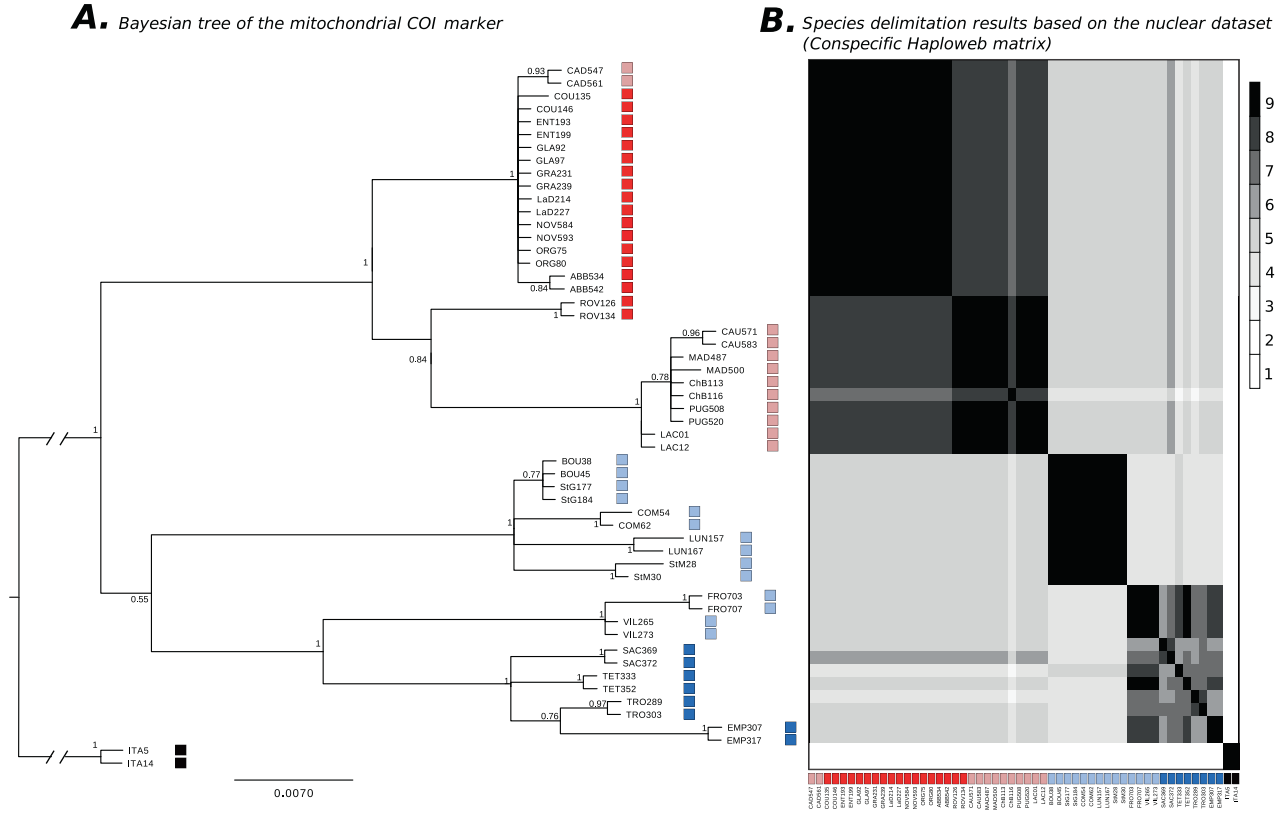

Fig. 2 Phylogenetic tree of the mitochondrial marker and conspecific matrix of the nuclear markers. A Bayesian tree based on the mitochondrial CO1 marker. Color boxes at the end of the tip correspond to the color of the geographical region of Fig. 1B. The two black boxes correspond to the two samples of the outgroup species *C. italica*; **B** Conspecific heatmap matrix based on the haploweb of the nine EPIC nuclear markers (Fig. S1). Localities are aligned in the same order as the phylogenetic tree. This heatmap matrix represents, for each pair of individuals, a score equal to the number of independent EPIC markers supporting the hypothesis that the two compared individuals belong to the same FFR ("fields for recombination", i.e., the same haplotype is co-occurring in heterozygous individuals) indicating by level of gray. Black reflects the highest scores (9 out of 9; the pair of individuals belong to the same FFR for all 9 EPIC markers) whereas the lowest scores (0 out of 9) are shown in white.

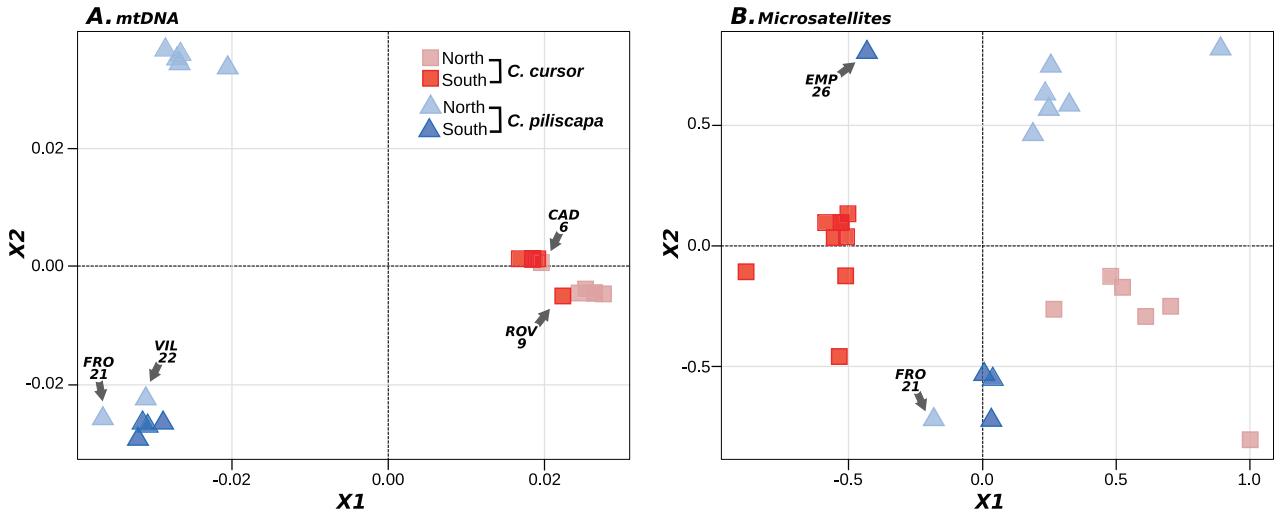

Fig. 3 Clustering analyses of the mitochondrial and microsatellite datasets. Nonparametric multidimensional scaling plot over two dimensions based on **A** the haplotypic distance (p-distance) for the mitochondrial DNA and **B** the Hedricks'Gst for the microsatellites data. Each point is a locality. The species is identified by the shape (triangle for *C. piliscapa* and square for *C. cursor*) and the region of origin by the same color than in Fig. 1B. The locality identity is given for those that do not cluster with the other localities of their region. The stress value of the NMDS was 3.67 for the mtDNA and of 20.92 for the microsatellite data.

were roughly equidistant. The microsatellites better discriminated the two regions within *C. cursor* than the mtDNA (Fig. 3). The proportion of private alleles between species was 39.5% (109 out of 276 alleles) with 48% of them found in a single locality and all except two occurring at low frequency (less than 13%). Interestingly, these two private alleles both found in *C. piliscapa* at high frequency (45% for one and 27% for the other) also discriminated the two regions of *C. piliscapa*, with the exception of VIL. For both species,

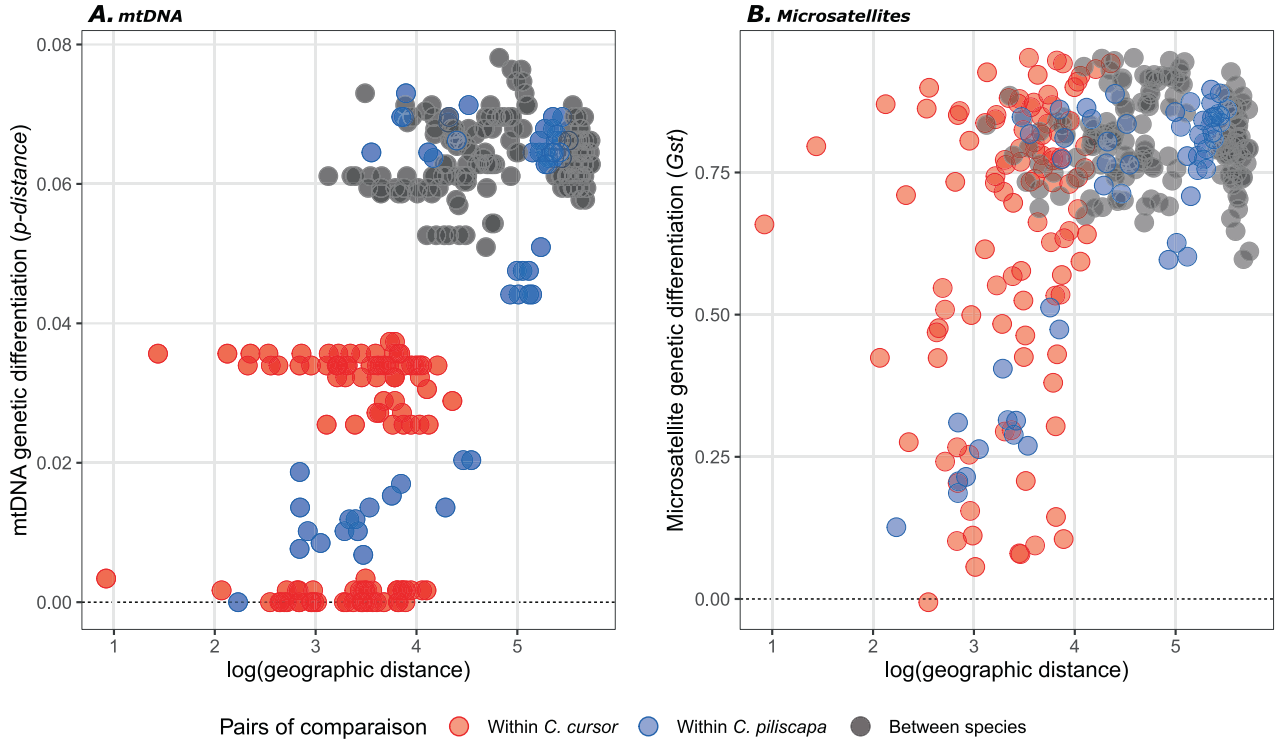

Fig. 4 Isolation by distance analyses of the mitochondrial and microsatellite datasets. Patterns of isolation-by-distance for **A** the mitochondrial marker based on the mean p-distance and **B** the microsatellite markers based on the Hedricks'G_{st}. Both are expressed on the logarithm of geographic distance. Pairs of localities of the same species are colored in red for *C. cursor* and blue for *C. piliscapa* whereas pairs comparing localities of the two species are black.

the proportion of private alleles between regions was higher than between the two species (within *C. piliscapa*: 50% (131/263) and within *C. cursor*: 50% (90/180)).

In agreement with the large level of pairwise genetic differentiation between localities, STRUCTURE analyses detected a large number of genetic clusters with the optimal k being 10; $K = 4$ being the second best solution (Fig. S2). At $K = 10$, the clusters identified by STRUCTURE were well delimited and consistent among runs. In all individuals, except one of *C. cursor* (from LAC), the highest level of assignment matched their region of origin and therefore species (Fig. S2). Only one locality of *C. cursor* (CAD), harbored a high number of individuals (12/15) where the largest assignment value was below 80%. Interestingly, this locality also showed discrepancy for the EPIC and mtDNA markers suggesting ongoing gene flow across the Durance River. Only two percent of individuals (7/369; five in *C. piliscapa* and two in *C. cursor*) had more than 20% assignment corresponding to populations of the other species. When forcing to $K = 4$, the four clusters defined by STRUCTURE were consistent among runs and corresponded to the four geographical regions (except for ROV). When forcing to $K = 2$, the results were not consistent among runs, with no clear biological interpretation of the results (Fig. S2). Overall, these results reflect the strong genetic differentiation between regions within species and among localities within a given region with low level of admixture (Fig. S2).

The partial Mantel test showed a significant positive association between pairwise G_{ST} and geographical distances after controlling for the presence of the Rhône River ($r = 0.32$; $P = 0.001$). Similarly, including the presence of the Rhône River separating pairs of localities significantly increased genetic differentiation after controlling for the geographical distances (partial Mantel test, $r = 0.18$; $P = 0.006$). This is graphically represented in Fig. 4B where interspecific comparisons always have high values of genetic differentiation (mean ± sd $G_{ST} = 0.79 \pm 0.06$) whereas intra-specific comparisons for the same geographical distances (between 20 and 50 km) vary from low to high values of genetic differentiation within both species (mean ± sd *C. cursor*: $G_{ST} = 0.65 \pm 0.25$; *C. piliscapa* $G_{ST} = 0.53 \pm 0.25$).

Chemical analyses

Our chemical analyses identified 44 peaks containing 74 compounds (Table S4). When training the RF analysis for discriminating the two species, the rate of individual misclassification to their group (OOB error rate) was very low (0.8%), showing that the species could confidently be identified from their cuticular hydrocarbons. Only two misclassifications (out of 239 individuals) were observed (Fig. 5), from the localities of StM and LUN. Training the RF to differentiate the two regions within *C. cursor* led to an OOB error rate of 5% (8 misclassifications out of 145 individuals; Table S12). The chemical analysis further segregated two clusters within *C. cursor*, which roughly correspond to the North and South regions identified using genetic markers. Note that our chemical analyses did not include individuals from the South region of *C. piliscapa* (see Materials and Methods). We observed a significant correlation between chemical and genetic distances (partial Mantel test, $r = 0.42$; $P = 0.005$) only due to the similar high genetic (based on G_{ST}) and chemical differentiation observed between localities of the two species (Fig. S3).

Morphometric analyses of workers

The clustering analysis revealed four different clusters based on both tibia size and the number of hairs (Fig. 6). Ninety-eight percent (147/150) of the individuals from clusters 1 and 2 belonged to *C. piliscapa* whereas 96% (210/219) of individuals from clusters 3 and 4 belonged to *C. cursor* (overall, twelve individuals were misclassified, Table S12). The clustering analysis confirmed that the two species can be discriminated with

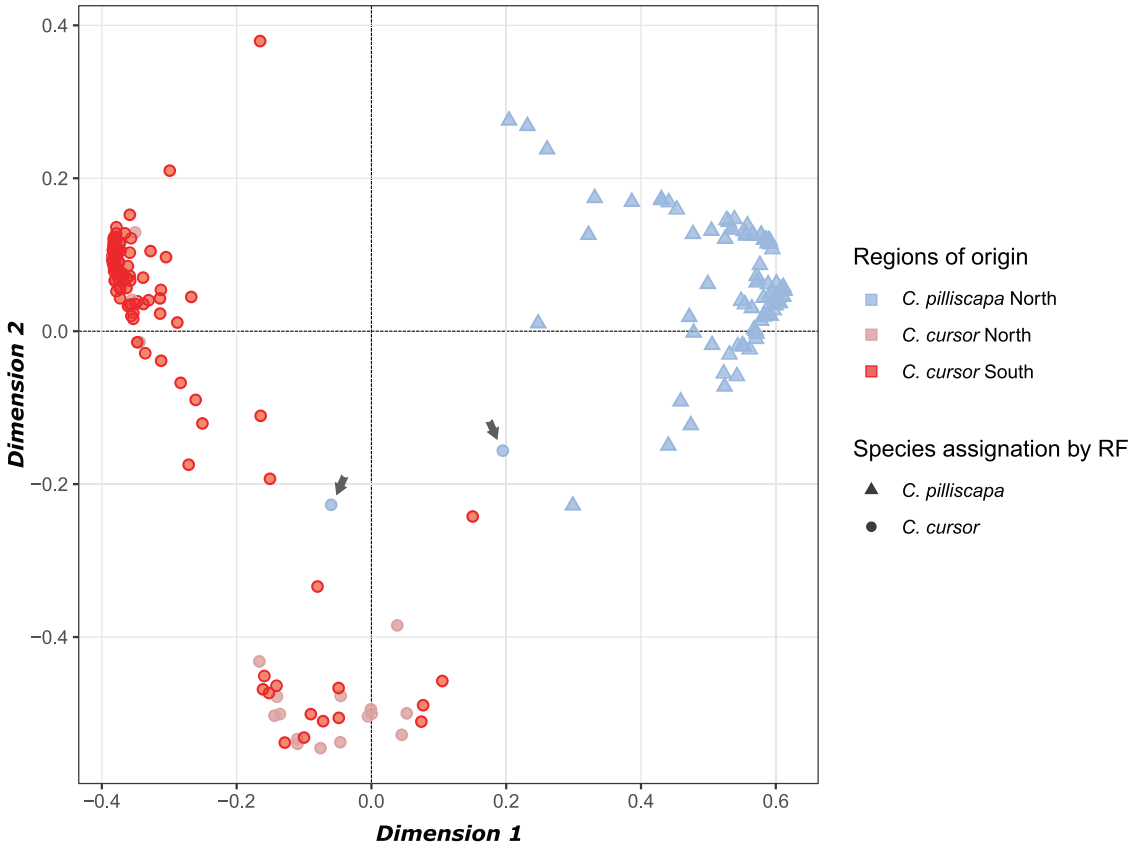

Fig. 5 Multidimensional scaling plot based on the cuticular hydrocarbon profile of workers. The metric multidimensional scaling plot was based on the pairwise proximity matrix between individuals estimated by the RF. When the classification of the RF is congruent with the origin of the individuals, the triangles (individuals classified as *C. piliscapa*) should be light blue (individuals originating from *C. piliscapa* North region), the circles (individuals classified as *C. cursor*) should be red (individuals originating from *C. cursor* North region) or pink (individuals originating from *C. cursor* South region). Note here that only two individuals noted with an arrow were misclassified into their species of origin by the RF.

relatively high accuracy based on the number of hairs, with the value of five representing the threshold between the two species. Considering only the number of hairs, 50% of individuals with five hairs (8/16) were wrongly assigned. Not surprisingly, in *C. cursor*, the three wrongly assigned individuals had five hairs and in *C. piliscapa*, the nine wrongly assigned individuals had either five, six or nine hairs. However, by averaging the number of hairs by locality, all localities were properly assigned (see Table S1 for the mean number of hairs per locality) suggesting that sampling many individuals from a given colony would allow a proper determination at the colony level. Within *C. piliscapa*, the two other clusters (1 and 2) determined by the analysis were discriminated by the tibia size with cluster 2 containing larger workers. However, they did not correspond to the two geographical regions since 52% and 63% of individuals from respectively clusters 1 and 2 belonged to the South region of *C. piliscapa*. Within *C. cursor*, the two clusters (3 and 4) were differentiated mainly by the number of hairs with cluster 4 having more hairs (Fig. 6). Interestingly, 82% of the individuals from cluster 4 belonged to the North region of *C. cursor*, whereas 74% of the individuals from cluster 3 belonged to the South region.

In agreement with the clustering analysis, the number of hairs significantly differed between the two species (L-ratio = 49.09, $P < 0.0001$) with an average of 0.97 (±1.77) hairs for *C. piliscapa* and of 12.95 (±5.16) for *C. cursor*. Although the differences are small, the two species also differed significantly by their tibia size (L-ratio = 9.27, $P = 0.002$), with *C. piliscapa* having longer legs (2.14 ± 0.20) than *C. cursor* (2.06 ± 0.17). Within each species, the number of hairs significantly differed between the two regions (*C. piliscapa*: L-ratio = 10.12, $P = 0.002$; *C. cursor*: L-ratio = 25.77, $P < 0.0001$) whereas the tibia size did not significantly differ (*C. piliscapa*: L-ratio = 0.21, $P = 0.64$; *C. cursor*: L-ratio = 0.02, $P = 0.96$).

Morphometric analyses of male genitalia

The shape of the four elements of the genitalia significantly differed between the two species (MANOVA: $P < 0.001$ for all the four elements; Fig. 7). This was especially clear for paramere and volsellae as the two species scattered along the two axes of the CVA (Fig. 7). The North and South regions of *C. cursor* differed in shape for volsellae ($P < 0.001$) and penisvalvae ($P < 0.001$), but not for paramere ($P = 0.51$) and sternum IX ($P = 0.529$). The North and South regions of *C. piliscapa* differed in shape for all the four elements (Sternum IX: $P = 0.003$; paramere: $P < 0.001$; volsellae: $P < 0.001$; penisvalvae: $P < 0.001$).

Behavioral experiments

The number of mating attempts was about twice higher with conspecific males than with heterospecific ones (L-ratio = 28.09, df = 1, $P < 0.001$), and in the absence of workers than in their presence (L-ratio = 6.77, df = 1, $P = 0.009$) (Fig. 8A, B). The interaction between the type of males and the presence of workers was not significant (L-ratio = 0.87, df = 1, $P = 0.35$). Overall, the number of mating attempts did not significantly differ between the gynes of the two species (L-ratio = 0.94, df = 1, $P = 0.33$). Interestingly, *C. cursor* and *C. piliscapa* males behaved differently towards heterospecific gynes. None of the six *C. cursor* males tried to mate with a *C. piliscapa* gyne, whereas three out of five *C. piliscapa* males tried to mate with a

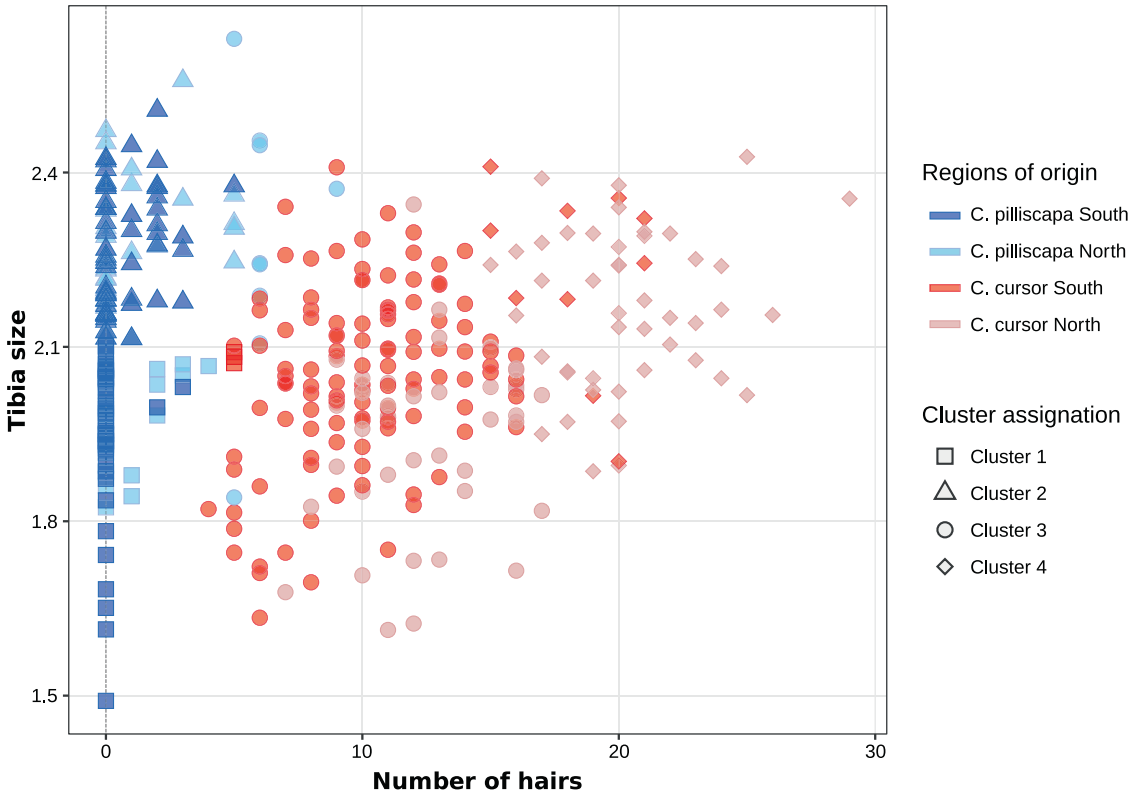

Fig. 6 Clustering based on the number of hairs (*X* axis) and tibia size (*Y* axis) of workers. Each point corresponds to an individual identified by the clusters it belongs to according to the clustering analysis (labeled from 1 to 4) and by the species (red or pink for *C. piliscapa* from the South and North region respectively and blue or light blue for *C. cursor* from the South and North region respectively). Clusters 1 and 2 correspond to *C. piliscapa* whereas clusters 3 and 4 correspond to *C. cursor*. The two species can clearly be discriminated based on the number of hairs. Misclassification can be observed for 12 individuals (cluster 1 or 2 classified as *C. cursor* (blue color) and cluster 3 classified as *C. piliscapa* (pink color).

C. cursor gyne (Fig. 8A, B, Table S5). As expected, males of both species attempted to mate with conspecific gynes (at least in the absence of workers). All except one *C. piliscapa* gynes mated with the conspecific male whereas no *C. cursor* gyne mated whatever the males (Fig. 8A, B, Table S5), even in absence of workers. Although this can result from the *C. cursor* gynes being not yet sexually mature (even though males tried to mate, which usually indicates that gynes were sexually mature too; C. Doums & T. Monnin, pers. obs.), it can also suggest that the conspecific males were too genetically dissimilar (they all originated from a different region whereas males were from the same region for *C. piliscapa* gynes).

Workers were more aggressive towards heterospecific individuals (males or workers) than towards conspecifics. However, the extent of the difference varied according to the type of individuals (males or workers) (significant interaction terms: L-ratio = 4.27, df = 1, $P = 0.04$, Fig. 8C, D). Even though both heterospecific males and heterospecific workers were significantly more aggressed than their conspecific counterparts (males: contrast estimate = 27.3, $t = 3.26$, df = 36.3, $P = 0.0024$; workers: contrast estimate = 51.4, $t = 6.13$, df = 36.3, $P < 0.001$), the extent of the difference was twice higher for workers (Fig. 8D) than males (Fig. 8C). The reason for this is that while conspecific foreign workers (Fig. 8D) were rarely aggressed, conspecific males were often victims of attacks (Fig. 8C), as previously observed (Helft et al. 2015). The only exception to this rule concerned one outcome where the conspecific workers suffered the highest level of aggression (Fig. 8D). Interestingly, this was the only test using a *C. piliscapa* gyne and a conspecific male originating from a different region and resident workers were very aggressive towards the two foreign workers, suggesting high aggression between regions in *C. piliscapa*. The workers of the two species did not significantly differ in their duration of aggression (L-ratio = 1.00, df = 1, $P = 0.32$).

DISCUSSION

We combined genetic, morphological, chemical, and behavioral analyses to assess the degree of differentiation between the sister ant species, *C. cursor* and *C. piliscapa* (Bondroit 1918; Agosti and Collingwood 1987) could be well differentiated. These two species were supposed to have a non-overlapping distribution on each side of the Rhône River (Nowbahari et al. 1990). We therefore specifically compared localities on each side of the river, assigning individuals to species according to their geographical origin in order to test independently each marker (even the taxonomic character). Our sampling was designed to maximize within-species differentiation by sampling localities either at the extreme ranges of the distribution (*C. piliscapa*) or on each side of a putative geographic barrier (the Durance River for *C. cursor*). Our results revealed that the between-species differentiation is only slightly higher than within-species differentiation, especially because of the high differentiation found between regions within species. Three markers, the mitochondrial DNA, the cuticular hydrocarbons, and the number of hairs, were able to independently distinguish the two species with no or low error rates whereas they were less successful in distinguishing the two regions within species (Table S12). Interestingly, we observed higher levels of aggression of workers toward heterospecific males that could lead to reproductive isolation between species by limiting mating attempts. No mating of heterospecific males was observed between species, even in absence of workers in our laboratory setting. Even though this result should be confirmed on a larger

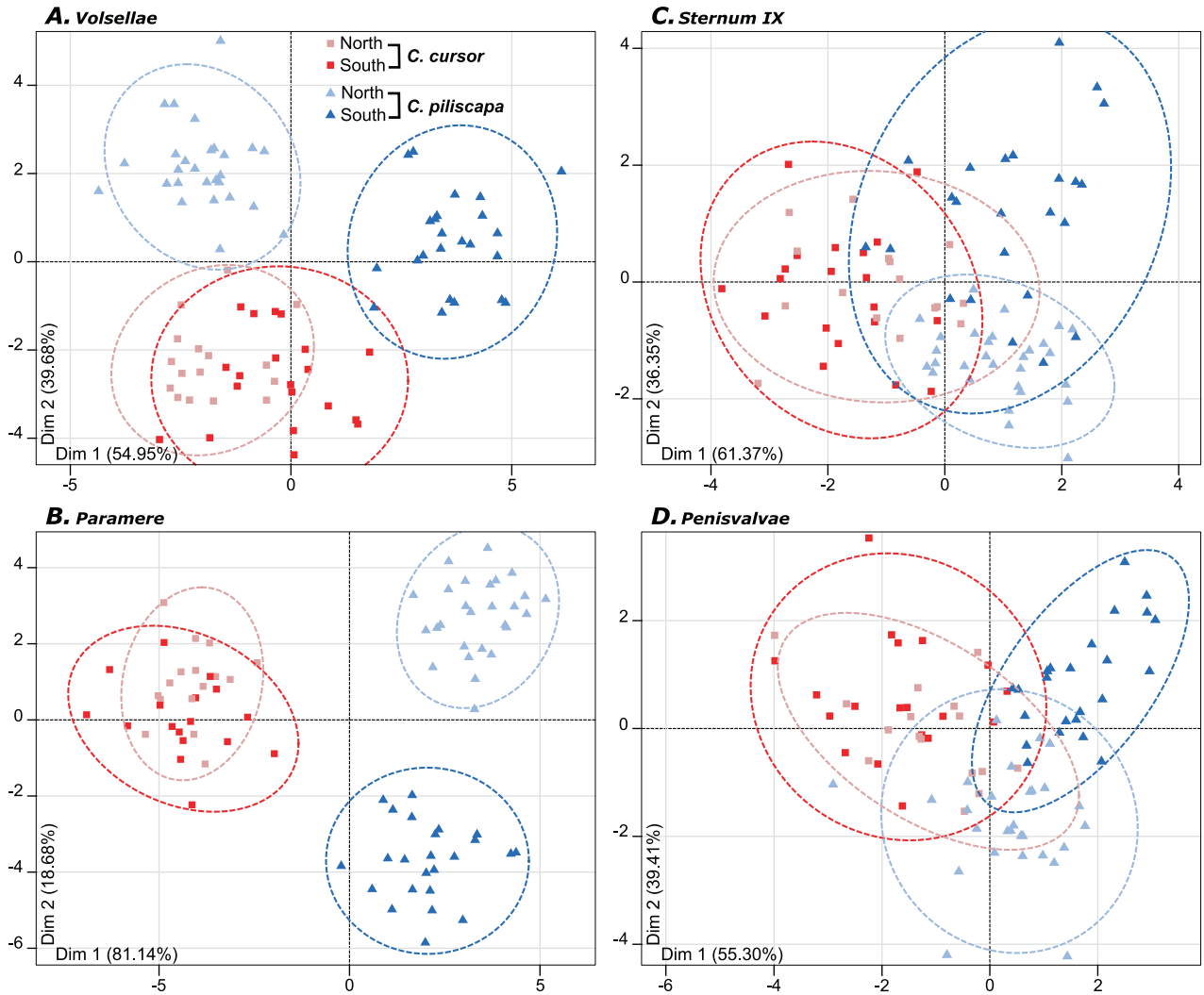

Fig. 7 First two axes of the canonical variate analysis for the four genitalia elements. A Volsellae **B** Paramere **C** Sternum IX and **D** Penisvalvae. Ellipses represent the 95% confidence intervals of the conformation average for each group.

sample size, they suggest reproductive isolation between the two species. Altogether, our results support the taxonomic separation of the two species even though the genetic integrity of the two species is still far from clear.

The three genetic markers do not offer a similar pattern even though all of them showed a slightly higher genetic differentiation between species than within species. MtDNA phylogenetic tree separated the two species; this marker, therefore, appears to be a promising marker for species identification, with five SNPs being species-diagnostic (Table S12), though additional sampling is needed to confirm that these SNPs are discriminant over all the distribution area. However, the genetic differentiation between the two species did not reach the level observed between them and the most closely related species *C. italica* (Fig. 2). Moreover, the level of genetic differentiation was only slightly higher between species (p-distance = 0.064) than between regions, at least for *C. piliscapa* (p-distance = 0.061). Among the nine nuclear EPIC markers studied, only a single SNP was found to discriminate the two species. These markers revealed three main clusters (Fig. 2), identifying two regions of *C. piliscapa* and a single cluster for *C. cursor*. Most EPIC markers shared at least one haplotype between the two species, which could reflect a lack of complete reproductive isolation or may stem from incomplete lineage sorting, whereby the low substitution rate of these genetic markers did not allow to accumulate enough divergence to fully segregate the two species. The microsatellite markers showed a more complex picture, where higher between-species genetic differentiation was only observed over short geographical distances (between 20 and 50 km, Fig. 4). In both species, the genetic differentiation was high between localities, even within regions, and could be as high as between species. Accordingly, ten well-delimited groups were detected by STRUCTURE and the two species could not be consistently distinguished for $K = 2$ (Fig. S2). For $K = 10$, few individuals in both species (2%) had more than 20% assignment corresponding to populations of the other species. Overall, the genetic markers therefore suggest that speciation is still ongoing between the two species and that the various levels of population genetic differentiation, even including those observed within each species, could represent pairs of populations at varying stages of the speciation continuum (Feder et al. 2012; Shaw and Mullen 2014; Stankowski and Ravinet 2021a, 2021b). However, our genetic markers were not enough powerful to confirm hybridization, and to disentangle current gene flow from shared ancestral polymorphism, as was done for instance by Cordonnier et al. (2019) in the ant *Tetramorium*. Hybridization is a common phenomenon in ants that could promote adaptive genetic changes (Kulmuni and Pamilo 2014; Martin-Roy et al. 2021) and is often associated with major changes in social organization and reproductive systems (i.e., Schwander et al. 2006; Eyer et al. 2016; Stolle et al. 2022).

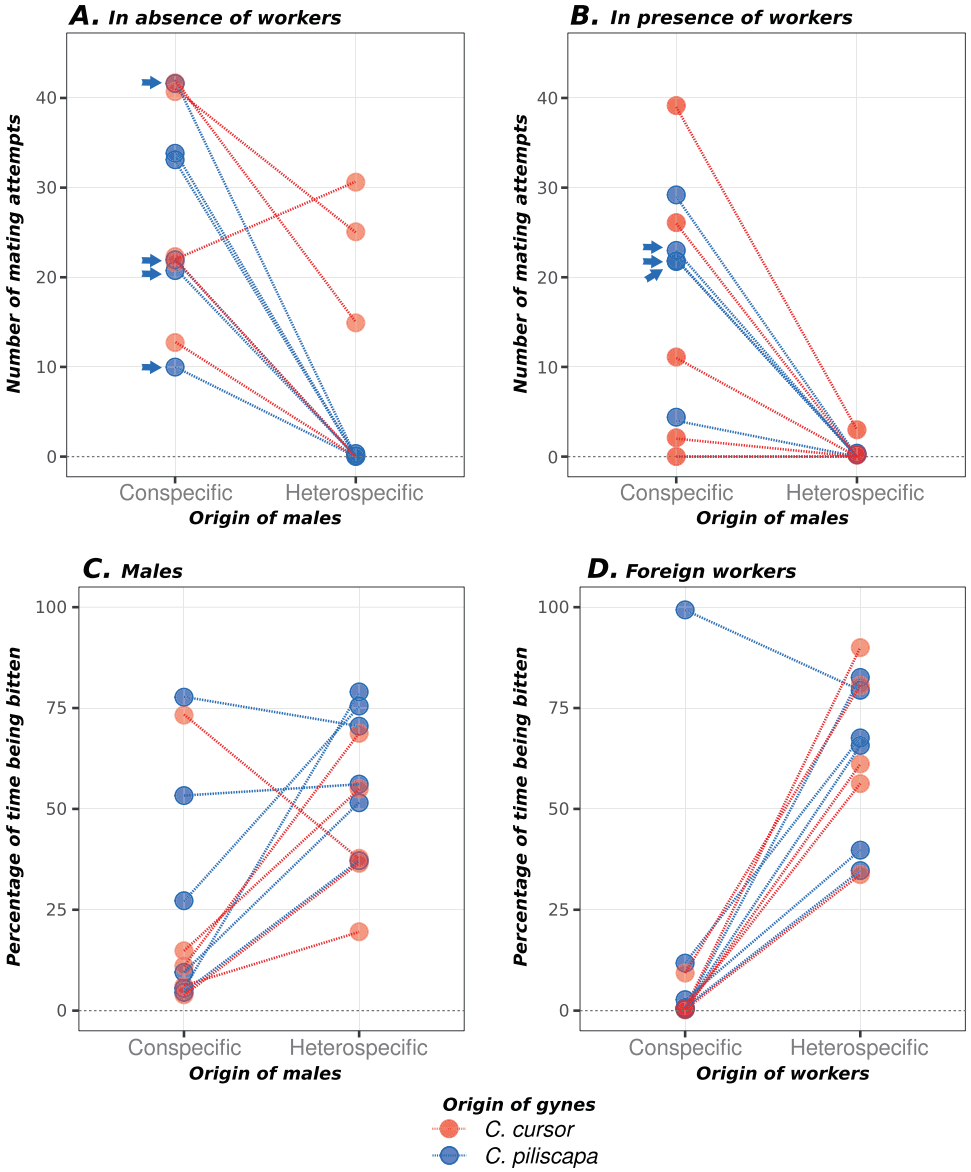

Fig. 8 Number ofmating attempts and aggression between conspecific and heterospecific individuals. For each of the 11 tests, the number of mating attempts by the conspecific and heterospecific males competing for the tested gyne is given in the absence of workers. **A** and in their presence **B**, as well as the percentage of time the introduced individuals (males (**C**) or foreign workers (**D**)) were bitten by resident workers. Each dot represents the performance of the tested individuals, the lines connecting the two males or two foreign workers used in the same test reflect an increasing or decreasing performance from conspecific to heterospecific. The color of the lines and dots indicates the species of origin of the gyne. For **A** and **B**, the arrows identify males with at least one successful mating (all arrows are blue indicating that all successful males were from *C. piliscapa*).

Whole genome analyses would be very promising in our study system to investigate these questions. They would also allow to identify putative genomic regions directly involved in reproductive isolation.

Morphological (number of hairs, size of workers, and male genitalia) and chemical (cuticular hydrocarbons) characters also showed a higher between-species than within-species differentiation. The taxonomic morphological criterion (number of hairs) can easily be used to identify the two species with low error rate (3%). Interestingly, our quantitative assessment of the threshold value (5 hairs) was the same than the one stated in the determination key by Agosti and Collingwood (1987). The chemical assessment was also successful in identifying the two species with a very low error rate at the individual level (0.8%). Interestingly, the misclassified individuals are not the same for the different criteria used, suggesting that the misclassification errors likely stem from variations within each criterion or are old-generation backcrosses. Cuticular hydrocarbon blends are used by social insects as cues to discriminate nestmates from non-nestmates. They are influenced by both genetic and environmental factors (Walsh et al. 2020) and are usually variable between and within species (Bos and D'Ettorre 2012; Sturgis and Gordon 2012; Eyer et al. 2021; Blumenfeld et al. 2021). Our results agree with other studies showing that chemical profiles can be used as relevant taxonomic tools in ants (Blomquist and Bagneres 2010; Seppä et al. 2011; Kather and Martin 2012; Villalta et al. 2018). The morphology of male genitalia is also frequently used as a taxonomic tool due to its high rate of evolution (Hosken and Stockley 2004; Simmons 2014; Kjer et al. 2016). However, although significant differentiation was detected between species (especially for the paramere), species distinction only based on this morphological character might be arduous unless using clustering analysis. The absence of

major morphological differentiation in male genitalia supports the idea that reproductive isolation during the speciation process could have been mainly driven by pre-copulatory mechanisms of sexual selection and/or mate recognition based on chemical signatures. This hypothesis deserves further tests in subsequent studies on this system.

Our behavioral experiments, even though based on limited sample size, suggest that the chemical differences detected between the two species are perceived, potentially with other morphological cues, and used by workers in the context of mating. Workers are usually not aggressive towards workers from other colonies, and foreign workers can be easily adopted if they do not come from geographically distant localities (Nowbahari and Lenoir 1989). On the contrary, workers belonging to the two distinct species are rapidly attacked and killed (Nowbahari et al. 1990, Fig. 8, Table S5). Our behavioral experiments using foreign workers as focal individuals confirm these results as they show stronger aggression towards heterospecific workers than conspecific ones. Similarly, heterospecific males were significantly more aggressed than conspecific males. Interestingly, the level of aggression towards conspecific males was higher than towards conspecific workers. This suggests that aggression towards males is specifically linked with the mating process rather than simply associated with worker colonial recognition. Our laboratory setting mimicking natural conditions ensures that these results reflect behavior occurring in natural populations. Moreover, similar aggressive behaviors of workers towards conspecific males from the same locality were already observed in a previous lab study (Helft et al. 2015) and on the field (Cronin et al. 2011; Doums and Monnin, pers. obs.). If workers preferentially aggress migrant males (i.e., heterospecific or from a distant locality), this indirect sexual selection could further limit male's contribution to gene flow and play a major role in population genetic differentiation and speciation. Sexual selection is known to be a major cause of reproductive isolation between species (Coyne and Orr 2004). To our knowledge, our results are the first line of evidence that this could be the case in a social insect species. Estimating the strength of pre-mating reproductive isolation and sexual selection (Rolán-Alvarez and Caballero 2000; Sobel and Chen 2014) for various pairs of populations differing by their level of genetic and phenotypic differentiation would shed light on the evolution of reproductive isolation at the different stages of speciation.

Our study also allows a better assessment of the population genetic structure of each species. Although this had been previously studied in *C. piliscapa*, this was only in the southern region (Clémencet et al. 2005), and nothing was known about *C. cursor*. The strong genetic structure observed in both species was expected given the mode of colony foundation by fission that reduces female gene flow by limiting colony dispersal to ant walking distances (few meters) and by diminishing the number of new colonies founded due to the high energetic cost in both putative species (Cronin et al. 2013). As observed in our study, this mechanism generally yields a pattern of isolation-by-distance among localities and high genetic differentiation between localities, consistent with what has been previously described in many ant species (Seppä and Pamilo 1995; Liautard and Keller 2001; Doums et al. 2002; Knaden and Wehner 2006; Leppänen et al. 2013). Even though flying males could erode the isolation-by-distance pattern induced by low female dispersal, they are not very good flyers (Hardy et al. 2008). Moreover, their contribution to gene flow can be ineffective if thelytoky is used at high frequency to produce new queens (Pearcy et al. 2004; Doums et al. 2013). The magnitude of genetic differentiation among populations within a species can be an indicator of the potential for speciation, as shown in vertebrates (Bernatchez and Wilson 1998) and insects (Ikeda et al. 2012; Polato et al. 2018). For instance, marine species with non-planktotrophic development and therefore with low dispersal show higher speciation rates than do planktotrophic forms (Hansen 1983). Whether colony fission and conditional parthenogenesis increase speciation rates in ants would merit further investigation.

The level of genetic diversity was high in both species even if slightly lower in *C. cursor* (Tables S9 and S10). Whether this reflects different reproductive strategies (use or not of thelytoky) awaits further comparative analysis. The high level of genetic diversity and allelic richness of the microsatellite markers could somehow be surprising under a high level of thelytoky (Pearcy et al. 2004; Doums et al. 2013) and low male dispersal (Hardy et al. 2008). Indeed, both mechanisms should increase the probability of mating between related males and females and subsequently lead to high level of inbreeding in workers. This discrepancy suggests that thelytoky might be lower than estimated directly based on gyne production in *C. piliscapa*. This could be due to a higher fitness of sexually produced gynes or to strong variation in the use of thelytoky over space and time not detected by our previous estimates (Pearcy et al. 2004; Doums et al. 2013). Moreover, indirect sexual selection by workers could also play a role in preventing inbreeding and/or outbreeding depression by being more aggressive towards highly related/differentiated males. Experiments are currently being conducted in the laboratory to test these hypotheses, assessing the spatial and temporal variation in the rate of thelytoky in the two species and testing for a cost of inbreeding on queen fitness.

The *C. cursor* species complex is therefore characterized by strong genetic differentiation not only between species but also among populations within species. The differentiation observed between species was only slightly higher than within species. However, it is not clear whether these patterns reflect genetic structure related to divergence time or isolation by distance without necessarily involving stronger reproductive isolation. By using an interdisciplinary approach, we found that the genetic differentiation between species is concomitant to morphological and chemical differentiation, as well as to an absence of mating between species and an elevated aggressiveness of workers towards heterospecific males. Behavioral experiments would be necessary within each species to test whether the strong genetic differentiation found between some pairs of populations, especially between regions, is associated with reproductive isolation. Our study, therefore, emphasizes that assessing phenotypic traits, antagonism, and mating attempt/success between individuals from distinct putative species is important in species with low dispersal and strong population structure to position the diverging populations along the speciation continuum. Species complexes with strong hierarchical genetic structures can provide ideal case studies of taxon pairs near the completion of speciation, which could ultimately help to understand the evolution of reproductive isolation along a speciation continuum (Stankowski and Ravinet 2021b).

Data archiving

DNA sequences: GenBank accession numbers MK684255 to 684308 (see Table S2). Microsatellite dataset has been deposited in the Open Science Framework database, https://osf.io (https://doi.org/10.17605/OSF.IO/Q6485).

Chemical, morphological, and behavioral data will be made accessible via DRYAD after acceptance.

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AUTHOR CONTRIBUTIONS

CD designed the studies. CD, TM, and PCB collected samples. PCB and PF performed the genetic experiments, BF and RC performed the morphometric experiments, PdE, CL, and TM performed the chemical experiments, BF and PCB performed the behavioral experiments. CD, PAE, BF, AK, and SM analyzed the data and all co-authors participate in the writing of the paper, with a major contribution of PAE, BF, and CD.

COMPETING INTERESTS

The authors declare no competing interests

ADDITIONAL INFORMATION

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