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SHORT COMMUNICATION

Genetic determination of female castes in a hybridogenetic desert ant

H. DARRAS, A. KUHN & S. ARON

Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium

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Abstract

In most social insects, the brood is totipotent and environmental factors determine whether a female egg will develop into a reproductive queen or a functionally sterile worker. However, genetic factors have been shown to affect the female's caste fate in a few ant species. The desert ant *Cataglyphis hispanica* reproduces by social hybridogenesis. All populations are characterized by the coexistence of two distinct genetic lineages. Queens are almost always found mated with a male of the alternate lineage than their own. Workers develop from hybrid crosses between the genetic lineages, whereas daughter queens are produced asexually via parthenogenesis. Here, we show that the association between genotype and caste in this species is maintained by a 'hard-wired' genetic caste determination system, whereby nonhybrid genomes have lost the ability to develop as workers. Genetic analyses reveal that, in a rare population with multiple-queen colonies, a significant proportion of nestmate queens are mated with males of their own lineage. These queens fail to produce worker offspring; they produce only purebred daughter queens by sexual reproduction. We discuss how the production of reproductive queens through sexual, intralineage crosses may favour the stability of social hybridogenesis in this species.

Introduction

Reproductive systems relying on the pairing between partners from different lineages or species have evolved multiple times in the animal kingdom and are far from being rare curiosity (Beukeboom & Vrijenhoek, 1998; Schlupp, 2005; Avise, 2008). Obligate hybridization is characteristic of hybridogenetic species, in which females of hybrid origin discard their paternal genome prior to meiosis and produce gametes carrying no paternally derived genes (Avise, 2008). The eggs are then fertilized with sperm of the paternal species resulting in a hybrid, which consists of a clonally inherited maternal part and a sexually inherited paternal part. As a consequence, in hybridogenetic species, both the maternal and paternal genomes are expressed in somatic tissues, whereas only the maternal genome constitutes the germ line and is perpetuated across generations.

Over the last decade, hybridogenesis has been reported at the social scale in several ant species: *Pogonomyrmex barbatus/rugosus* species complex (Helms Cahan *et al.*, 2002; Julian *et al.*, 2002), *Solenopsis xyloni* × *Solenopsis geminata* (Helms Cahan & Vinson, 2003), *Wasmania auropunctata* (Fournier *et al.*, 2005), *Vollenhovia emeryi* (Ohkawara *et al.*, 2006; Kobayashi *et al.*, 2008), *Paratrechina longicornis* (Pearcy *et al.*, 2011) and *Cataglyphis* spp. (Leniaud *et al.*, 2012; Eyer *et al.*, 2013). Hybridogenetic ant populations are characterized by the co-occurrence of two interbreeding but distinct genetic lineages. The offspring of a male and a queen of different lineages develop into sterile workers, whereas pure-lineage offspring develop into new reproductive offspring (new queens and males). Social hybridogenesis therefore results in a situation in which the 'soma' of the colony is hybrid, whereas the reproductive lines carry pure-lineage genomes. Hybridogenesis has direct consequences on caste determination in ants, that is whether a female egg will

Correspondence: Hugo Darras, Evolutionary Biology and Ecology, CP 160/12, Université Libre de Bruxelles, 50, av. F.D. Roosevelt, 1050 Brussels, Belgium.
Tel.: +32 2 650 22 67; fax: +32 2 650 24 45;
e-mail: hgdarras@gmail.com

develop into a reproductive queen or a sterile worker. In eusocial Hymenoptera, the female brood is usually totipotent and caste determination is generally under environmental control (Wheeler, 1986). In contrast, social hybridogenesis leads to a strong caste–genotype association among females. For example, in *Pogonomyrmex* hybrids and *W. auropunctata*, the association between genotype and caste has been shown to be maintained by a genetic caste determination (GCD) system, whereby nonhybrid genomes have lost the ability to develop as workers (Helms Cahan *et al.*, 2004; Clark *et al.*, 2006; Schwander *et al.*, 2006; Volny *et al.*, 2006; Foucaud *et al.*, 2010). GCD is assumed in other hybridogenetic ants as well (Smith *et al.*, 2008; Schwander *et al.*, 2010), but still remains to be demonstrated by breeding evidence.

Here, we provide direct evidence of genetic determination of female castes in the hybridogenetic ant *Cataglyphis hispanica.* In this species, two bisexual lineages (*H1* and *H2*) co-occur as a complementary pair across the whole distribution range of the species (Leniaud *et al.*, 2012; Darras *et al.*, 2014). In most populations, colonies are headed by a single queen mated once with a male originating from the alternate genetic lineage than their own. They produce sterile hybrid workers by sexual reproduction, but they use parthenogenesis for the production of reproductive offspring. As a consequence, the genetic lineages form two separate gene pools that do not exchange any genetic material. However, a recent genetic survey of colony structure across the distribution range of *C. hispanica* revealed rare populations with multiple-queen colonies (Darras *et al.*, 2014). Although all queens carried alternate lineage sperm in populations with single-queen colonies, queens carrying same-lineage sperm were found occasionally in populations with multiple-queen colonies. We first investigated the significance of intralineage mating in a population with multiple-queen colonies. We found that a significant proportion (15%) of mated queens store same-lineage sperm. Second, we compared the reproductive output between inter- and intralineage mated queens. Our results show that the queens mated with males from the same lineage fail to produce worker offspring. Nevertheless, intralineage mated queens can produce purebred queens by sexual reproduction. This caste system differs from the one known thus far for other *C. hispanica* populations, where only parthenogenesis has been reported for queen production.

Materials and methods

Collection of colonies

The ant *Cataglyphis hispanica* is found in the most arid habitats of the south-west of the Iberian Peninsula. In *Cataglyphis*, there is no overwintering brood. Male and female sexuals develop from eggs laid during the first days after queens resume egg laying in early spring, whereas all eggs laid later in the season are reared into workers (Cagniant, 1979; Aron *et al.*, 2011). Eighteen colonies were completely excavated in Cáceres (Spain), in a population with both single-queen and multiple-queen colonies (mean number of queens ± SD per colony with multiple queens: × ± SD = 6.4 ± 4.1; Table 1). Collection was performed in April 2013, before queens resumed egg laying.

Genetic caste determination

Production of queens maintained in experimental single-queen colonies

To investigate the reproductive output of interlineage and intralineage mated queens, we conduced blind experiments. We settled 62 experimental colonies, each containing a single queen of unknown mating status (mated with a male of the same genetic lineage, mated with a male of the alternate lineage, or unmated) and 200 workers from the field. Colonies were maintained under laboratory conditions and fed on maggots and sugar water. They were surveyed twice a week for 2 months. The caste of offspring (daughter queen, male, or worker) was determined upon emergence from pupae and then preserved in pure ethanol.

Egg mortality

After producing a first batch of reproductive individuals, 12 of 62 colonies stopped to rear any offspring, while others were readily producing worker offspring (see Results). We tested whether the lack of larvae in some colonies, but not in others, was related to differential egg mortality among queens. To this aim, we first removed all brood from 8 experimental colonies producing workers and eight colonies with no worker larvae. The following week, all newly laid eggs were frozen at −20 °C for subsequent genetic analyses. The proportion of dead eggs was estimated following the method of Schwander *et al.* (2006), which is based on the percentage of PCR-amplifiable eggs. DNA was extracted from eggs using a modified Chelex extraction protocol (Clark *et al.*, 2006) and amplified at eleven microsatellite loci with fluorescent primers and Qiagen Type-it kit for multiplex PCR of microsatellite loci [*Cc11*, *Cc54*, *Cc93*, *Ch01*, *Ch05*, *Ch06*, *Ch08*, *Ch11*, *Ch12*, *Ch22* and *Ch23* (Pearcy *et al.*, 2004; Darras *et al.*, 2014)]. PCR products were run on an ABI 3730 sequencer. Fluorescent signals were visualized using GENEMAPPER v. 3.5 (Applied Biosystems, Foster City, CA, USA). Dead eggs have degraded DNA and cannot be successfully amplified (Schwander *et al.*, 2006). Eggs with allele peak heights over 400 relative fluorescence units (RFUs) were considered alive.

Table 1 Colonies excavated, queens found in each colony, mating status of queens given as 'queen lineage/sperm lineage' ('?'indicates no data; '–'indicates a virgin queen), number of offspring of each caste and sex produced, and proportion of eggs that could be successfully amplified.

			Number of offspring produced			
Colony	Queen	Mating status	Workers	New queens	New males	PCR-amplifiable eggs
A01	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	
	3	H2/ ?				
	4*	H2/H2	–	15	3	3/15
	5*	H2/H1	> 12	–	–	
	6	H2/H1				
	7	H2/H1				
	8	H2/H1				
A02	1*	H1/H2	> 12	–	–	
A04	1*	H2/H1	> 12	–	–	
A05	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	
	3*	H2/H1	> 12	–	–	
A06	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	1	10/15
	3*	H2/H1	> 12	–	–	
	4*	H2/H1	> 12	–	–	
A07	1*	H2/H1	> 12	–	–	
A08	1*	H1/H2	> 12	–	–	
A09	1*	H2/H2	–	5	–	3/15
	2*	H2/H1	> 12	–	–	
	3*	H2/H1	> 12	–	–	
A10	1*	H2/H1	> 12	–	1	
A11	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	15/15
A12	1*	H1/H2	–	–	–	
A13	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	
	3*	H2/H1	> 12	–	–	
	4*	H2/H1	> 12	–	–	
A14	1*	H2/H1	> 12	–	–	15/15
A15	1*	H2/H1	> 12	–	–	15/15
	2*	H2/H1	> 12	–	–	
	3*	H2/H1	> 12	–	–	
	4*	H2/H2	–	–	-	4/15
	5*	H2/H1	> 12	–	–	
	6*	H2/H2	–	2	–	1/15
	7*	H2/H2	–	–	1	4/15
	8*	H2/H1	> 12	–	–	
	9*	H2/H2	–	15	6	5/15
	10*	H2/H2	–	2	–	0/15
	11	H2/H1				
	12	H2/H2				
C1	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	
	3*	H2/H2	–	1	–	1/15
	4*	H2/H1	> 12	–	–	
	5*	H2/ –	–	–	2	
	6*	H2/H1	–	–	–	
	7*	H2/H1	> 12	–	–	
	8*	H2/H1	> 12	–	–	
	9*	H2/H1	> 12	–	–	
	10*	H2/H1	> 12	–	–	
	11*	H2/ –	–	–	–	
	12*	H2/H1	> 12	–	–	
	13*	H2/H1	> 12	–	–	
	14*	H2/H1	> 12	–	–	
C2	1*	H2/H1	> 12	–	–	11/15
	2*	H2/H1	> 12	–	–	9/15
	3*	H2/H1	> 12	–	–	
	4*	H2/H1	> 12	–	–	13/15
	5	H2/H1				
	6	H2/H1				
	7	H2/H2				
	8	H2/ –				
C3	1*	H2/H1	> 12	–	–	
	2*	H2/H1	> 12	–	–	
	3*	H2/H1	> 12	–	–	
	4*	H2/H1	> 12	–	–	
	5*	H2/H1	> 12	–	–	
	6*	H2/H1	> 12	–	–	
C4	1*	H1/H2	> 12	–	–	13/15

*Queens reared in experimental colonies for investigation of reproductive output.

Significance of intralineage mating

Frequency of intralineage mated queens

At the end of the experiments, we determined the mating status of queens (mated or unmated) and the proportion of queens mated with males of their own or the alternate lineage. The queens were killed, their abdomen was dissected in a Ringer's solution, and the content of their spermathecae was examined. A queen was considered virgin when its spermatheca was empty. If she was mated, we determined the origin of her male partner by comparing the genotype of the queen with the genotype of the sperm stored in the spermathecae. DNA was extracted from the queens by adding 100 μL 5% Chelex to a leg sample and incubating at 85 °C for 2 h. Sperm DNA was extracted following a similar procedure with 20 μL 5% Chelex. Eleven microsatellite loci were amplified as previously described for eggs (see above). Allele scoring was carried out using GENEMAPPER v. 3.5. The lineage memberships of the queens and their partners were determined based on their position on a principal coordinates analysis (PCoA) built on genetic distances and including published genotypes of known lineages as a reference (Darras *et al.*, 2014).

Mode of production of new queens by intralineage mated queens

In all populations of *C. hispanica* with single-queen colonies sampled to date, queens were always found mated with a male of the alternate lineage than their own (Leniaud *et al.*, 2012; Darras *et al.*, 2014). Workers are hybrids of the two lineages, whereas new queens are pure-lineage individuals produced by thelytokous parthenogenesis. We examined whether new queens can also develop from intralineage crossing in populations with multiple-queen colonies. Females produced by intralineage sexual reproduction would indeed bear pure-lineage genomes and are predicted to develop into queens if the female caste is genetically determined. To this aim, we determined the proportion of new daughter queens arising from parthenogenesis or from intralineage mating in the experimental colonies. DNA of new queens was extracted and amplified at the 11 microsatellites loci. New queens were considered parthenogenetically produced if they harboured alleles that can all be attributed to their mother. They were considered sexually produced if they bore one allele of their mother's mate (whose genotype was determined from sperm sampling, see above) at all loci. Relatedness coefficient among nestmate queens was estimated using the program RELATEDNESS 5.0.8 (Queller *et al.*, 1989).

Results

From the 18 colonies sampled, eight contained a single queen and 10 hosted multiple queens (Table 1). In multiple-queen colonies, queen number per colony varied from 2 to 14; the mean relatedness among nestmate queens was 0.90 (SD = 0.14). In total, we collected 72 queens. As expected from previous studies (Darras *et al.*, 2014), the genotypes of the queens and their mates formed two clusters on the PCoA (Fig. 1) consistent with the co-existence of two genetic lineages in the population. Four queens belonged to the *H1* lineage ($n = 4$ colonies), whereas the other 68 queens belonged to the *H2* lineage ($n = 14$ colonies). All queens from lineage *H1* originated from single-queen colonies, whereas queens from lineage *H2* were found in both single-queen and multiple-queen colonies ($n = 4$ and 10 colonies, respectively). We were able to dissect all but one queen. Sperm was found in 68 spermathecae; the remaining three spermathecae were empty, indicating that the queens were unmated. All mated queens had sperm from a single male: 58 queens (85%) were mated with a male from the alternate lineage than their own, and 10 queens (15%) had sperm from a male of their own lineage. The intralineage mated queens and unmated queens belonged to the *H2* lineage only.

From the 62 experimental colonies set-up for the monitoring of queen reproductive output, *a posteriori* genetic analyses revealed that 52 were headed by a queen mated with a male of the alternate lineage (interlineage mated queen), eight colonies were headed by a queen mated with a male of the same lineage (intralineage mated queen), and two colonies contained virgin queens. All 62 queens laid eggs during the 2 months of the experiment. However, there were dramatic differences in brood development among the three types of queens (Table 1). Fifty of the 52 experimental colonies headed by an interlineage mated queen produced worker offspring. By contrast, none of the colonies headed by an intralineage mated queen ($n = 8$) or a virgin queen ($n = 2$) produced any workers (Fisher's exact test on the proportion of colonies producing workers, intra- vs. interlineage mating: $P < 0.001$). An opposite trend was found regarding sexual production. Colonies headed by interlineage mated queens reared only two males (not a single new queen was found), whereas colonies with intralineage mated queens produced a total of 10 males and 40 new queens. All the new queens produced arose from sexual (intralineage) reproduction, not parthenogenesis. One experimental colony headed by a virgin queen (colony C1 – queen 5) produced two males (Table 1). All the males reared harboured alleles that belong to the queen lineage, indicating that they were queen's sons. Overall, sexual productivity was significantly higher in colonies with intralineage mated queens than in colonies with interlineage mated queens (Fisher's

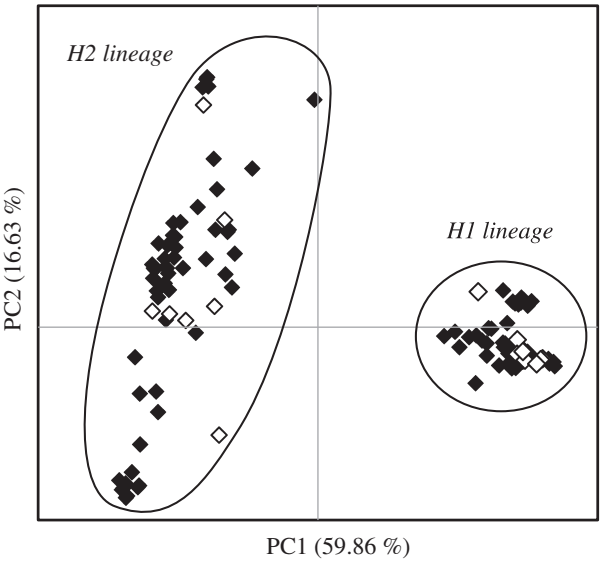

Fig. 1 Plot of the first two axes from the PCoA based on genetic distances between reproductive individuals of *Cataglyphis hispanica*. Filled diamonds: genotypes of the queens and their mating partners (i.e. inferred from the content of the spermatheca) in Cacéres (Spain); open diamonds: reference data for the same population are taken from Darras *et al.* (2014).

exact test on male production: $P = 0.014$; on queen production: $P < 0.001$).

We compared the mortality of eggs laid by eight interlineage mated queens and eight intralineage mated queens at the period of worker production, through PCR amplification trials. The percentage of eggs successfully amplified (i.e. alive) was significantly higher for interlineage mated queens (0.84; range: 0.67–1) than for intralineage mated queens (0.17; range: 0.0–0.33) (Student's *t*-test, $t_{14} = 9.52$, $P < 0.001$). All the eggs genotyped arose from sexual reproduction.

Discussion

Our data provide three novel insights into our understanding of social hybridogenesis in *Cataglyphis* desert ants. First, they show that 15% of mated queens store same-lineage sperm, indicating that no strong barriers to assortative mating exist in the population of Cacéres where half the colonies are headed by multiple queens. Our estimate of the frequency of intralineage mating may even be underestimated because it is based on mother queens mated during past reproductive seasons. As intralineage mated queens do not produce workers and cannot found new colonies by their own, they might experience higher mortality than interlineage mated queens during their early life. Thus, the ratio of intra- vs. interlineage mated queens reported here provides a biased estimate of the strength of putative prezygotic barriers to assortative mating. Likewise, in hybridogenetic populations of *Pogonomyrmex*, colonies are headed by a single-queen mated multiple times with males of their own as well as with males of the alternate lineage. The initial colony foundation success of queens is dependent on their stock of interlineage sperm (for worker production) relative to intralineage sperm (for queen production) (Helms Cahan *et al.*, 2004; Anderson *et al.*, 2006; Schwander *et al.*, 2006).

Second, our study provides direct evidence for a genetic caste determination (GCD) system in the desert ant *C. hispanica*. They show that nonhybrid eggs have lost the ability to develop as workers. In contrast with interlineage mated queens whose fertilized eggs (*H1/H2*) always developed into workers, eggs of *H2* queens fertilized by same-lineage sperm only developed into new queens; not a single *H2/H2* egg laid at the period of worker production reached the larval stage. Selective elimination of pure-lineage brood after the period of sexual production seems highly unlikely. Intralineage mated queens laid approximately the same amount of eggs as interlineage mated queens, and their eggs were kept in normal piles without visible signs of destruction. Moreover, previous studies have shown that ant workers do not recognize the sex/caste of the eggs, but of the larvae (Aron, 2012). Rather, our genetic analyses strongly support the view that intralineage *H2/H2* eggs laid after the period of sexual production suffered intense mortality compared to hybrid eggs. One explanation could be that intralineage eggs develop into queens only when triggered by maternal clues, at the beginning of the reproductive season. Maternal effects were indeed reported in various ant species, where queens were shown to exert substantial proximate control over the caste fate of their eggs by nutritionally or hormonally biasing their development into a queen or a worker form (Passera & Suzzoni, 1979; Helms Cahan *et al.*, 2004; De Menten *et al.*, 2005; Schwander *et al.*, 2008; Libbrecht *et al.*, 2013). Unfortunately, our data do not allow testing for the plasticity of H1/H1 pure-lineage offspring. Although, consistent with GCD in both *H1* and *H2* lineages, the association between caste and genotype is perfect in all populations; all 1228 workers genotyped so far were hybrids, whereas all 304 queens had pure-lineage genotypes (Leniaud *et al.*, 2012; Darras *et al.*, 2014; our study). Genetic caste determination was also demonstrated in hybridogenetic *Pogonomyrmex* and in *Wasmannia auropunctata* (Helms Cahan *et al.*, 2004; Clark *et al.*, 2006; Schwander *et al.*, 2006; Volny *et al.*, 2006; Foucaud *et al.*, 2010). However, the genetic bases of caste determination remain unknown in these ants; several genetic models have been proposed, but none has received experimental support so far (Sirviö *et al.*, 2011).

In *Cataglyphis hispanica*, post-mating selection may explain (i) the complete absence of intralineage mated queens in single-queen populations, but (ii) its presence in multiple-queen populations (Leniaud *et al.*, 2012; Darras *et al.*, 2014). (i) In single-queen populations, queens establish a new colony at a walking distance of their natal nest with the help of a worker force (Leniaud *et al.*, 2012). The helping workers may kill or abandon an intralineage mated queen that is unable to produce her own worker offspring and regain their natal nest. In the conspecific species *Cataglyphis cursor*, where single queens also found new colonies by budding, it has been shown that a new nest can be reabsorbed by the parent colony if it proves to be unviable (Cheron *et al.*, 2011; Cronin *et al.*, 2012). If assortative mating was frequent in populations with single-queen colonies, this process could explain why no intralineage mated queens are found in their mature nests. (ii) In multiple-queen populations, queens can either establish a new colony by budding or be readopted by their natal colony, where they may cooperate with their mother and sisters. In line with this, our genetic analyses show that the relatedness among nestmate queens is very high (0.90). In contrast with what likely happens in single-queen colonies, workers of multiple-queen colonies may not reject promptly low-quality queens as other queens can ensure colony survival. Hence, an intralineage mated queen may persist in a multiple-queen colony, where she can produce female and male sexuals without contributing to the worker force. Such brood parasitism

may either be a best of a bad situation strategy or a 'cheater' strategy. In the first scenario (best of a bad situation), a female mated with a 'wrong' male gains nonnull fitness by joining a multiple-queen colony instead of founding alone. In the second scenario, brood parasitism is considered as a form of cheating expressed by males. Cheater males would directly benefit from mating with a female of the same lineage as they can enhance their fitness by fathering reproductive daughters (new queens) through intralineage sexual production of queens, whereas noncheating males only sire workers and have null fitness.

Third, this study shows that queens of *C. hispanica* can produce new daughter queens by classical sexual reproduction between partners from the same lineage. In all populations sampled so far, new reproductive queens were found produced by thelytokous parthenogenesis (Leniaud *et al.*, 2012; Darras *et al.*, 2014). Quite surprisingly, none of our experimental colonies produced new queens by parthenogenesis. Whether queens did not lay parthenogenetic eggs or whether thelytokous eggs did not reach the adult stage remains uncertain. Previous study showed that only few queens lay thelytokous eggs and that the proportion of these eggs laid is usually very small (Aron *et al.*, 2011). In colonies headed by intralineage mated queens, the high production of female sexuals was likely an experimental artefact due to pure-lineage brood being 'hard-wired' to develop into reproductive individuals rather than workers. The frequency of queens arising from intralineage sexual reproduction under natural conditions remains to be examined.

Importantly, the production of queens through sexual, intralineage reproduction may play a key role in the stability of social hybridogenesis in *C. hispanica*. First, the development of new reproductive individuals by parthenogenesis keeps males with null fitness expectancies. The production of males fathering worker offspring only has been suggested to be a threat for the stability of social hybridogenesis in *Cataglyphis*, because queens would be expected to stop the production of a sex with null fitness (Schwander & Keller, 2012). The present work shows that males occasionally father reproductive daughters and, hence, that they are not evolutionary dead ends. Second, and in a more general context, asexual lineages are predicted to be evolutionary short lived because asexual reproduction is associated with long-term fitness disadvantages (Williams, 1975; Smith, 1978; Bell, 1982). The occasional production of new reproductive queens by sexual reproduction therefore provides an escape from the dead end of pure asexuality.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Microsatellite genotype data are available in the electronic supplementary material (genotypes_genepop.txt).

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