

Intraspecific queen parasitism in a highly eusocial bee

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Insect societies are well-known for their advanced cooperation, but their colonies are also vulnerable to reproductive parasitism. Here, we present a novel example of an intraspecific social parasitism in a highly eusocial bee, the stingless bee *Melipona scutellaris*. In particular, we provide genetic evidence which shows that, upon loss of the mother queen, many colonies are invaded by unrelated queens that fly in from unrelated hives nearby. The reasons for the occurrence of this surprising form of social parasitism may be linked to the fact that unlike honeybees, *Melipona* bees produce new queens in great excess of colony needs, and that this exerts much greater selection on queens to seek alternative reproductive options, such as by taking over other nests. Overall, our results are the first to demonstrate that queens in highly eusocial bees can found colonies not only via supersedure or swarming, but also by infiltrating and taking over other unrelated nests.

Keywords: social parasitism; reproductive conflict; stingless bees; *Melipona scutellaris*

1. INTRODUCTION

Insect societies are well-known for their advanced cooperation, but their colonies can also be exploited by interspecific and intraspecific social parasites which can benefit from the resources stored within the nest, and get directly cared for by their hosts. Recently, social bees have become a major focus in the study of social parasitism in insect societies, after the discovery of several novel, highly unusual cases of intraspecific worker parasitism in this group [1]. For example, it was shown that both bumblebee [2] and queenless honeybee colonies [1,3] are occasionally parasitized by workers from other nests that fly in and lay male-producing eggs, which are then reared by the victim colony. In addition, in the Cape bee *Apis mellifera capensis*, where workers can produce female offspring via thelytokous parthenogenesis, a single clonal lineage of worker bees was found to reproductively parasitize and kill colonies of African

honeybees, *Apis mellifera scutellata* [1,4]. One study even found the Cape bees to occasionally lay female-destined eggs directly into queen cells, thereby reincarnating themselves as queens [5].

In contrast to these varied forms of intraspecific social parasitism reported for bumblebees and honeybees, as yet little is known about the occurrence of such social parasitism in the other major group of eusocial bees, the stingless bees. Recently, however, Sommeijer *et al.* [6,7] speculated that intraspecific queen parasitism might perhaps occur in the stingless bee genus *Melipona*, after observing that in *Melipona favosa*, a large percentage of the virgin queens (57%) left their natal nest and that lone queens apparently tried to enter and take over other unrelated colonies nearby. Indeed, there are good *a priori* reasons for expecting such intraspecific queen parasitism in *Melipona*. In contrast to other highly eusocial bees, queens in *Melipona* are reared in great excess of colony needs, with *ca* 5–25% of all females developing as queens [8–10]. This phenomenon is linked to the fact that in *Melipona*, queens and workers develop in identical, sealed brood cells on a similar provision mass, thereby allowing females to control their own caste development and causing many to develop as queens, with the hope of being able to head a new swarm or replace a failing mother queen [11,12]. Nevertheless, chances of doing so successfully are slim, and the majority of all queens are normally killed by the workers soon after emergence [13–15], or are dispelled out of the colony [7,16]. With such low chances of any one queen succeeding in founding or inheriting a nest, penetrating and taking over other unrelated colonies nearby would thus be expected to be a profitable alternative to gain reproductive benefits [6].

The aim of this study was to carry out the first formal genetic test of whether or not intraspecific queen parasitism occurs in stingless bees, and whether queens could indeed succeed in entering unrelated hives to opportunistically rear their own brood. In order to do so, we carried out a long-term genetic study on the Brazilian stingless bee, *Melipona scutellaris*, and sampled female brood before and after queen replacement events to check whether newly established queens were either the daughter of the previous queen, or instead were unrelated queens that had flown in from elsewhere.

2. MATERIAL AND METHODS

To study the incidence of intraspecific queen parasitism, we monitored 10 free-foraging *M. scutellaris* colonies in the bee laboratory at the University of São Paulo for a period of *ca* 3 years and regularly checked all colonies for queen replacement events (figure 1). Via genotyping at three microsatellite loci, we then determined whether the replacement queens were daughters of the previous queen or alien queens that had flown in from other hives nearby (detailed methods can be found in the electronic supplementary material). This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated (*cf.* [17]), as is typical for stingless bees [18]. Indeed, the probability of misclassification was very low, 0.004 (see the electronic supplementary material). We also artificially induced eight queen replacement events and obtained data on a further six queen replacement events from colonies kept in an apiary at São Simão, 200 miles north. In all cases, the multi-locus genotypes allowed alien queens to be unambiguously traced back to a particular source colony (figure 1; electronic supplementary material, table S1).

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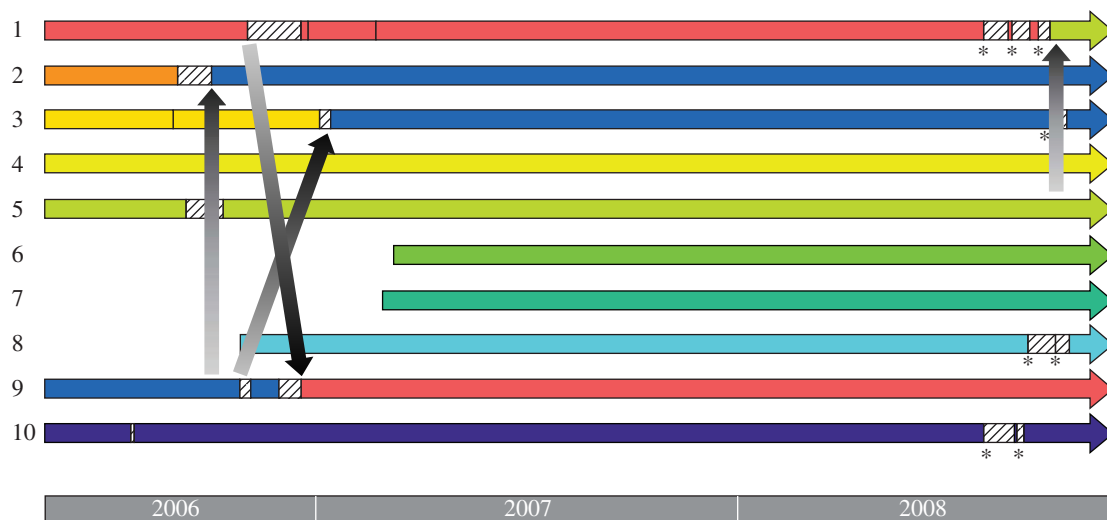


Figure 1. Intraspecific colony takeover by unrelated queens in the 10 *M. scutellaris* colonies kept in São Paulo. Coloured blocks show the tenure of any one queen; hatched areas are periods during which the colonies became queenless, after which a new queen was adopted. Asterisks indicate experimental queen removals. Vertical arrows indicate cases where genotyping showed newly adopted queens to be unrelated queens that had flown in from other hives nearby. The order of the colonies on the figure corresponds to the relative positions at which they were placed in the laboratory.

3. RESULTS

The queen genotypes (electronic supplementary material, table S1) show that intraspecific queen parasitism was common in *M. scutellaris*, with 25 per cent of all queen replacements (six out of 24) being with an alien queen that had flown in from another colony (95% binomial confidence limits: 9.8–46.7%). Colony takeovers by alien queens were detected both in the colonies kept in the laboratory in São Paulo (three out of ten natural queen replacements and one out of eight induced queen replacements, figure 1) as well as in the colonies kept in the apiary of São Simão (two out of six natural queen replacements; electronic supplementary material, table S1), where colonies were kept at a lower density (*ca* five colonies per hectare), akin to that found in nature. In all these cases, the new queens had genotypes that did not match with the genotype expected if they would have been the daughters of the superseded queens, and relatedness estimates show that the newly adopted alien queens were not related to either the adopting workers (mean $r = -0.07$, 95% C.L.: $(-0.27, 0.14)$, $n = 6$; electronic supplementary material, table S1) or the superseded queens (mean $r = -0.14$, 95% C.L.: $(-0.53, 0.24)$, $n = 6$). Alien queens also never came from neighbouring colonies (figure 1). This means that alien queen takeovers could not have been a mere by-product of queens accidentally returning to the wrong hive after their mating flight. In fact, in two out of four of the colonies in São Paulo, we inferred that the alien queens definitely came from queenright source colonies (the queen from colony 9 invading colony 2 and that of colony 5 invading colony 1, figure 1), and such colonies would normally not be expected to send out queens for mating.

The median queen life-expectancy in our study colonies was 175 days (Kaplan–Meier analysis, $n = 20$) and it took on average 15 days for a dead queen to be replaced (range 0–46, $n = 18$; electronic supplementary material, table S1). This means that at

any one time, 8.6 per cent (15/175) of the colonies in the population would find themselves queenless, at which point they would be vulnerable to be invaded by alien queens. There was no significant difference between the time that it took for a dead queen to be replaced by a daughter queen (14.9 days, s.d. 13.7, $n = 14$) and by an alien queen (16.8 days, s.d. 9.3, $n = 4$; t -test, $t = 0.25$, $p = 0.81$). This means that colonies that had remained queenless for a long time were not more likely to adopt alien queens. Colonies also continuously produced a large number of virgin queens (*ca* 50 during the 15 days that it took for colonies to re-queen). Even considering the fact that workers may kill some of the virgin queens before allowing one to leave on a mating flight (*ca* seven out of eight in *Melipona quadrifasciata* [13]), it is therefore clear that colonies did not adopt unrelated queens merely out of a shortage of natal queens.

4. DISCUSSION

Our results convincingly demonstrate, based on genetic data from two different localities, that *M. scutellaris* queens can leave the hive and infiltrate and successfully take over unrelated, queenless hives nearby. Given that Sommeijer *et al.* [6,7] collected behavioural evidence suggesting that the same phenomenon may be happening in another related species, *M. favosa*, it is probable that such queen parasitism occurs in many more *Melipona* species. This gives credibility to intraspecific queen parasitism being a specific reproductive strategy that evolved in response to the vast queen overproduction that occurs in this genus [8–10] and which selects queens to seek reproductive opportunities outside their natal colony. More generally, our data provide the first solid evidence that queens in highly eusocial bees can found colonies, not only via queen supersedure or swarming, but also by infiltrating and taking over unrelated nests nearby.

Previously, in social bees, anecdotal evidence for queens entering and taking over unrelated nests of the same species was found only in some species of primitively eusocial bumblebees, where late-emerging queens occasionally usurp and take over conspecific nests in the colony-founding stage [19–21], and in African honeybees, *A. m. scutellata*, where swarms sometimes usurp weaker colonies of the European honeybee [22]. Nevertheless, it is clear that both phenomena are quite different from the one we document, with the first being restricted to the colony-founding stage, and the second involving whole swarms of bees invading colonies of a related subspecies, as opposed to lone queens infiltrating and parasitizing conspecific nests in *Melipona* [6].

The occurrence of intraspecific queen parasitism in *Melipona* may well have important evolutionary consequences. For example, if queens are able to successfully invade unrelated nests, then producing new queens may become a profitable way for the adult workers to export copies of their own genes to the rest of the population. In fact, this may explain why in *M. favosa* and *Melipona compressipes*, workers have been observed to actively chase virgin queens out of the colony, resulting in about half leaving the colony alive [7,16]. That some gynes still end up being killed may be due to the workers perceiving the gynes as a threat to the current queen, since gynes have been found to occasionally attack the mother queen—presumably to try to kill and replace her [15]. The fact that producing many queens may genetically benefit colonies is in contrast to previous models [11,12], which argued that from a colony-level perspective, queen overproduction in *Melipona* always represents a great cost. On the other hand, it is true that there should also be strong selection for queen parasitism to be kept at a low level, given that workers should be selected to prevent unrelated queens from invading their colony. Providing such events are rare, however, accepting an occasional unrelated parasite queen may not entail a big cost, since the cost of accidentally rejecting a daughter queen would probably be much larger. In addition, it has been shown that *M. scutellaris* workers may keep on producing their own sons until many months after a new queen has become established, thereby providing them with direct fitness benefits even if they would accept an unrelated queen [17]. Given that virgin queens were continuously produced in high frequency, we consider it unlikely, however, that workers were adopting unrelated queens merely in situations when related queens did not happen to be available, that is, in order for them to be able to continue producing their own sons and ‘make the best of a bad situation’. Future work will have to determine how common intraspecific queen parasitism is in other species of stingless bees, including in natural, unmanaged populations.

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ELECTRONIC SUPPLEMENTARY MATERIAL

SUPPLEMENTAL METHODS

Study species

Melipona scutellaris nests in cavities of tree trunks in the Atlantic rainforest and is widely distributed in the North-east of Brazil [1], where it is commonly kept by regional and traditional beekeepers for honey, pollen and wax [2], and is also increasingly used for the pollination of various tropical crops [3, 4]. Colonies are perennial and swarm-founded, are headed by one singly-mated queen and typically contain around 1,500 workers [5-7].

Colony maintenance

Between May 2006 and March 2007 10 *Melipona scutellaris* colonies, each headed by a newly mated queen, were obtained from a beekeeper in Igarassu (Pernambuco state, Brazil, 7°50'3.74"S 34°54'22.87"W). Upon arrival these colonies were put in free-foraging nest boxes in the bee laboratory at the University of São Paulo (São Paulo state, 23°33'53.75"S 46°43'52.19"W), where they were placed ca. 1 metre apart, with colonies 1 to 3 placed along the outside wall of the lab and colonies 4 to 10 along an adjoining wall, at a right angle to the first (the order of the colonies corresponds to that given in figure 1). In addition, we obtained data from 6 *M. scutellaris* colonies that were part of a larger population of 39 hives kept at a farm ("Aretuzina") in São Simão (São Paulo state, 21°26'25.97"S 47°34'54.65"W). Colonies in this population were spaced ca. 5 to 10 metres apart, as is typical in most apiaries and also not uncommon in nature, where both in this as well as in other *Melipona* species, several nests can sometimes be found in aggregations within a few metres of each other, often in the same tree [8, 9]. For example, in *M. quadrifasciata*, it has been reported that 20% of all trees that are nested in contain more than one colony [9]. The overall nest density (number of hives divided by the size of the bee yard) was ca. 20 colonies/ha for the colonies kept in São Paulo and ca. 5 colonies/ha for those kept in São Simão. Natural nest densities in *Melipona* are generally a bit lower, usually in the range of 1 to 4 nests/ha [2, 9]. Nevertheless, the relatively high nest density in our apiaries does not imply that opportunities for queens to infiltrate and take over nests was much greater than in nature: assuming that gynes in *Melipona* have a similar radial flight range as workers and males, ca. 1 km [10], gynes in natural populations would have access to about 314-942 colonies in their local neighbourhood. By contrast, our study colonies were placed in apiaries located in São Paulo state, where this species does not naturally occur, which meant that gynes only had access to the available study colonies - 10 and 39 hives in São Paulo and São Simão, respectively.

Monitoring of queen replacement events

In order to determine the timing of queen replacement events, we marked all mother queens with a coloured plastic tag (in colonies kept at the laboratory) or white paint dot (in colonies kept at the farm) on their thorax, and regularly checked all queens over a total period of ca.

3 years (daily for the colonies kept in the lab and once every two weeks for the colonies kept at the farm). Before and after each queen replacement event, we sampled 10 worker pupae per colony and, whenever possible, also collected a wing tip of the mother queen (table S1), which were preserved in absolute ethanol for later genotyping. To further boost our sample size, we artificially induced eight queen replacement events in four colonies placed at the bee laboratory, whereby we manually removed the mother queen and waited for a new one to be adopted (figure 1, table S1). Again, wing tips of all these queens were preserved in absolute ethanol for later genotyping.

Genetic analysis

For parentage analysis we genotyped 10 worker pupae and either a wing tip sample from a live queen or a leg if she was killed or had recently died for each of our 16 colonies (table S1) at 3 microsatellite loci, *T4-171* [11] and *Mbi-254* and *Mbi-201* [12] (cf. [13]). Loci were reasonably polymorphic, with a total of 6, 8 and 7 alleles detected in both populations, and expected heterozygosities of 77%, 72%, 73% (in S. Paulo) and 51%, 64%, 61% (in S. Simão) at loci, *T4-171*, *Mbi-254* and *Mbi-201*, respectively. DNA was extracted using the Chelex method, whereby a single leg (from worker pupae or mother queen) or wing tip sample (from mother queens) was frozen in liquid nitrogen and ground up using a plastic pestle, followed by an incubation at 95°C for 15 min in 200µL (50µL for wing tip samples) of a 10% Biorad Chelex 100 resin solution. Samples were vortexed and centrifuged before use. Multiplex PCR reactions were carried out in a 10µL reaction volume, and contained 0.5µM of the forward and reverse primers of each locus, 0.2mM of each dNTP, 1.5mM MgCl₂, 1µL of crude DNA extract, 0.4 units of Silverstar Taq polymerase (Eurogentec, Seraing, Belgium) and enzyme buffer supplied by the manufacturer. PCR was performed following a touch-down programme [14], with an initial denaturation for 3min at 94°C, followed by 20 cycles consisting of 30s at 94°C, 30s at 58°C, but decreasing 0.5°C in each step, and 45s at 72°C; 10 cycles consisting of 30s at 94°C, 30s at 46°C, and 45s at 72°C; and a final 10-min extension step at 72°C. After amplification, 1µL of the PCR product was mixed with 8.8µL formamide and 0.2µL Genescan 500 LIZ size standard (Applied Biosystems, Lennik, Belgium), denatured, and loaded onto an ABI-3130 Avant capillary sequencer. Alleles were called using the supplied Gene Mapper software and manually checked.

The incidence of colony take-overs by alien queens

To test whether the genotypes of newly established queens were consistent with them being the daughters of the previous queen or being alien queens that had flown in from other hives nearby, we first reconstructed the genotype of each mother queen and that of her mate from the genotypes of the worker brood (for a detailed description of this method see [15]). This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated, as is typical for stingless bees [5]. In many cases, we also had the genotype of the queen herself available, which further facilitated par-

entage reconstruction (table S1). When mother queens were genotyped, the queen genotype was also always identical as the one which would have been inferred from the worker genotypes only. To eliminate the possibility of genotyping errors, any samples suggesting supersedure by an alien queen were regenotyped twice. Since *M. scutellaris* does not naturally occur in São Paulo state, all queens could be tracked down unambiguously to one of our laboratory colonies. The mean probability of erroneously classifying a queen as the daughter of the previous one when it was in fact an alien one in the São Paulo population was very low, 0.0040 (it was 0 for 12 out of 14 inferred supersedures by daughter queens, and 0.0313 and 0.0250 for queens SP 01-c and SP 03-d respectively, see table S1).

Relatedness of alien queens to adoptive colonies

To test whether the alien queens that took over some of the colonies were unrelated to the adoptive colonies, we estimated the relatedness between the alien queens and the adoptive workers as well as to the superseded queens using *Relatedness 5.0.4* [16]. Separate population allele frequency estimates were provided for the São Paulo and São Simão populations, and were calculated based on a previous genetic study of both populations [13]. Standard errors and 95% confidence limits on the relatedness estimates were calculated by jackknifing over loci [16].

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ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1. Colonies used in this study with data on sampling date, reconstructed parental genotypes, queen longevity, dates at which new queens became established, whether or not colonies were taken over by non-natal queens and the relatedness of non-natal queens to the adoptive workers (estimated using *Relatedness*).

Colony and queen	Sampling date	Parental genotypes at locus			Queen longevity (days)	Date that queen became established	# of days it took for the previous queen to be replaced	Colony takeover by a non-natal queen? (number of diagnostic loci)	Colony origin of alien queen	Relatedness of alien queen to adopting workers ($\pm SE$)
		T4-171	Mbi-254	Mbi-201						
<i>a) Parental genotypes of São Paulo colonies over the period that natural queen replacements occurred</i>										
SP 01-a ^a	10/2006	102/108 x 102	205/222 x 207	147/153 x 159	175	12/05/2006	-	-		
SP 01-b ^{a,b}	12/2006	102/108 x 104	205/207 x 197	153/159 x 153	7	19/12/2006	46	no		
SP 01-c ^{a,c}	02/2007	104/108 x 102	197/205 x 191	153/153 x 150	59	26/12/2006	0	no		
SP 01-d ^a	07/2008	102/108 x 108	191/205 x 205	150/153 x 150	>525 ^e	23/02/2007	0	no		
SP 02-a ^a	07/2006	102/104 x 104	197/207 x 205	144/153 x 153	115	12/05/2006	-	-		
SP 02-b ^a	02/2007	104/108 x 104	205/205 x 207	153/153 x 150	>760 ^f	03/10/2006	29	yes (2)	SP 09-a	0.38 \pm 0.18
SP 03-a	07/2006	102/104 x 104	205/207 x 207	147/159 x 159	111	12/05/2006	-	-		
SP 03-b	08/2006	102/104 x 102	207/207 x 207	147/159 x 153	127	31/08/2006	0	no		
SP 03-c ^a	07/2007	104/106 x 108	197/205 x 222	153/153 x 147	>624 ^e	14/01/2007	9	yes (3)	SP 09-a	-0.20 \pm 0.34
SP 04-a	07/2007	100/102 x 100	197/205 x 205	147/159 x 159	>904 ^f	12/05/2006	-	-		
SP 05-a ^a	06/2006	100/102 x 100	207/207 x 205	159/159 x 159	122	12/05/2006	-	-		
SP 05-b	11/2006	100/102 x 102	205/207 x 207	159/159 x 159	>750 ^f	13/10/2006	32	no		
SP 06-a	10/2008	102/106 x 108	200/211 x 205	153/153 x 153	>603 ^f	09/03/2007	-	-		
SP 07-a	10/2008	100/102 x 100	200/205 x 205	150/153 x 156	>611 ^f	01/03/2007	-	-		
SP 08-a	12/2006	104/110 x 104	191/205 x 197	150/153 x 144	>681 ^e	28/10/2006	-	-		
SP 09-a	08/2006	106/108 x 104	197/205 x 205	153/153 x 153	169	12/05/2006	-	-		
SP 09-b ^a	01/2007	104/106 x 108	197/205 x 222	153/153 x 147	24	06/11/2006	9	no		
SP 09-c	09/2008	102/108 x 102	205/207 x 203	147/159 x 140	>683 ^f	19/12/2006	19	yes (3)	SP 01-a	-0.25 \pm 0.44
SP 10-a	07/2006	102/108 x 102	205/207 x 205	150/153 x 153	74	12/05/2006	-	-		
SP 10-b ^a	04/2007	102/108 x 102	205/205 x 207	150/153 x 153	>735 ^e	28/07/2006	3	no		

b) Genotypes of queens that were newly adopted following experimental queen removal (São Paulo)

SP 01-e ^a	10/2008	108/108	205/205	150/153	(>3) ^{e,§}	22/08/2008	21	no		
SP 01-f ^a	10/2008	108/108	191/205	150/150	(>7) ^{e,§}	10/09/2008	16	no		
SP 01-g ^a	10/2008	102/102	205/205	159/159	(>35) ^{f,§}	27/09/2008	10	yes (1)	SP 05-b	-0.001±0.25
SP 03-d ^{a,d}	10/2008	106/108	205/222	147/153	(>19) ^{f,§}	13/10/2008	14	no		
SP 08-b ^a	10/2008	104/110	197/205	144/150	(>1) ^{e,§}	01/10/2008	23	no		
SP 08-c ^a	10/2008	104/104	191/197	144/153	(>19) ^{f,§}	13/10/2008	11	no		
SP 10-c ^a	08/2008	102/102	205/207	153/153	(>1) ^{e,§}	28/08/2008	27	no		
SP 10-d ^a	08/2008	102/102	205/207	150/153	(>57) ^{f,§}	05/09/2008	7	no		

c) Parental genotypes of São Simão colonies that underwent natural queen replacements

SS 02-a ^a	07/2006	104/106 x 106	205/211 x 197	159/159 x 153						
SS 02-b	02/2007	106/108 x 106	205/205 x 197	153/153 x 153				yes (3)		-0.34±0.21
SS 06-a	08/2006	104/108 x 106	205/211 x 197	150/153 x 150						
SS 06-b ^a	12/2006	106/108 x 108	197/211 x 211	150/150 x 153				no		
SS 08-a ^a	03/2006	108/108 x 106	205/205 x 211	150/159 x 153						
SS 08-b ^a	10/2006	106/108 x 106	205/211 x 197	150/153 x 159				no		
SS 36-a	03/2006	106/108 x 108	197/205 x 205	150/150 x 153						
SS 36-b	11/2006	106/108 x 108	205/205 x 205	150/153 x 150				no		
SS 59-a	09/2006	106/108 x 106	197/205 x 211	153/153 x 159						
SS 59-b ^a	01/2007	106/106 x 106	197/205 x 211	153/153 x 150				yes (2)		0.005±0.21
SS 63-a	04/2006	106/108 x 106	197/205 x 211	153/159 x 153						
SS 63-b ^a	11/2006	106/106 x 106	205/211 x 205	153/153 x 159				no		

^a Maternal genotype obtained by direct genotyping of the mother queen (using either a wing tip sample from a live queen or a leg if she was killed or had recently died). ^b Since this queen laid eggs for only 7 days no brood could be sampled for genetic analysis. However, on 26/12/2006 the mother queen was found dead in the colony and so could be collected for genetic analysis. Given that the genotype of queen 1-d could not have originated from any other colony, we know via elimination that she was the daughter of 1-c, and this allowed us to indirectly infer the genotype of the male that queen 1-c had mated with. ^c Maternal genotype was consistent with it being the daughter of 1-b, but with a probability of 1/4 it could also have been the daughter of 9-a. Given that at that time there were 8 alien colonies from which an unrelated queen could have originated, the probability of queen 1-c being erroneously classified as a daughter of 1-b is $1/4 \times 1/8 = 0.031$. ^d Maternal genotype was consistent with it being the daughter of 3-c, but with a probability of 1/4 it could also have been the daughter of 9-b. Given that at that time there were 10 alien colonies from which an unrelated queen could have originated, the probability of queen 3-d being erroneously classified as a daughter of 3-c is $1/4 \times 1/10 = 0.025$. ^e Queen experimentally removed. ^f Queen still alive on 1/11/2008. ^g Since queens were experimentally removed or only recently became established the estimates were not used in the calculation of median life expectancy.