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Asexual queen succession mediates an accelerated colony life cycle in the termite *Silvestritermes minutus*

R. FOUGEYROLLAS,*[1] J. KŘIVÁNEK,†‡[1] V. ROY,* K. DOLEJŠOVÁ,†‡ S. FRECHAULT,* Y. ROISIN,§ R. HANUS† and D. SILLAM-DUSSÈS¶**

*Université Paris-Est Créteil, Université Pierre et Marie Curie, CNRS, INRA, IRD, Université Paris-Diderot, iEES-Paris, 61 avenue du Général de Gaulle, 94010 Créteil, France, †Chemistry of Social Insects, The Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Flemingovo n. 2, 166 10 Prague, Czech Republic, ‡Faculty of Science, Charles University in Prague, Albertov 6, 128 43 Prague, Czech Republic, §Evolutionary Biology and Ecology, Université Libre de Bruxelles, Av. F. D. Roosevelt 50, 1050 Brussels, Belgium, ¶IRD-Sorbonne Universités-UMR 242, Université Pierre et Marie Curie, Université Paris-Est Créteil, Université Paris-Diderot, CNRS, INRA, iEES-Paris, 32 avenue Henri Varagnat, 93143 Bondy, France, **LEEC, EA4443, Université Paris 13-Sorbonne Paris Cité, 99 Avenue J. B. Clément, 93430 Villetaneuse, France*

Abstract

Mixed modes of reproduction, combining sexual processes with thelytokous parthenogenesis, occur in all major clades of social insects. In several species of termites, queens maximize their genetic input into nondispersing replacement queens through parthenogenesis, while maintaining genetically diverse sterile offspring and dispersing reproductives via sexual reproduction. This so-called asexual queen succession (AQS) has multiple independent origins and its presumed advantages are diverse as well, ranging from multiplication of colony reproductive potential to extension of its lifespan beyond that of the foundress. However, how AQS shapes colony life cycles under natural conditions remains poorly known. The neotropical termite *Silvestritermes minutus* inhabits small but conspicuous nests, offering a unique opportunity to investigate the impact of AQS on life history. We report on its breeding system, life cycle and sex allocation using social structure census in 137 nests and genotyping of 12 colonies at 12 microsatellite loci. We show that colonies are established by an outbred pair of primary reproductives. In less than 2 years, the foundress is replaced by multiple neotenic queens, arising mostly through automixis with central fusion. Sterile castes, male and most (93%) female dispersers are produced sexually. Colony reproduction is usually restricted to a single dispersal of alates with unbiased sex ratio, taking place after 3 years. We conclude that *S. minutus* benefits from AQS to maximize colony growth rate and alate production within a very short life cycle rather than to extend colony lifespan. This highlights the versatile role of AQS in different cases of its polyphyletic origin.

Keywords: asexual queen succession, breeding system, life history, parthenogenesis, *Silvestritermes minutus*, termites

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Introduction

Sexual reproduction is a widespread, yet not universal mode of reproduction in eukaryotes. Its indisputable evolutionary dominance is complemented by a multitude of asexual processes, distributed across a wide range of taxa (Bell 1982). However, only in rare cases

Correspondence: Robert Hanus, Fax: +420 220 183 578; E-mail: robert@uochb.cas.cz

[1]These authors contributed equally to this study.

was the sexual reproduction completely lost. Instead, a variety of mixed strategies evolved, combining asexuality and sex to reduce the obvious costs inherent to sexual reproduction (Maynard Smith 1978; Barton & Charlesworth 1998; Schön *et al.* 2009). In recent years, mixed modes of reproduction alternating sexual reproduction with thelytokous parthenogenesis have been identified to be stable elements of the life cycle in social insects as well, including the major eusocial clades, ants and termites (Wenseleers & Van Oystaeyen 2011).

The most noteworthy example of sexual reproduction combined with thelytoky has been documented in several species of ants, in which the queens produce the workers using the conventional sexual process from fertilized eggs, while new queens arise from unfertilized eggs through thelytokous parthenogenesis (reviewed in Wenseleers & Van Oystaeyen 2011; Rabeling & Kronauer 2013). This unusual reproductive system is interpreted as an ideal compromise between sexual and asexual reproductions, allowing the ant queens to maintain the desirable genetic diversity in workers while maximizing their genetic contribution to future queens and thus overcoming one of the major drawbacks of sex, the dilution of genetic material in each sexually produced generation (e.g. Pearcy *et al.* 2004).

As yet another fascinating example of convergent evolution between ants and termites, the mixed mode of reproduction has also been recently reported in the latter group. It is now known as asexual queen succession (AQS) and was first observed by Matsuura *et al.* (2009) in the Japanese subterranean termite *Reticulitermes speratus* (Rhinotermitidae), in which the founding primary queen is replaced by numerous neotenic females produced from unfertilized eggs through thelytokous parthenogenesis. The sterile colony members (workers and soldiers), as well as the large majority of winged dispersing reproductives, which are future colony founders, are produced sexually through the mating of the primary king with the primary queen or her parthenogenetic daughters.

The main adaptive significance of AQS in *R. speratus* colonies was proposed to be the maximization of the genetic input into next queen generation(s), while maintaining the desirable genetic diversity of sterile offspring and fertile winged dispersers. Queen replacement by large numbers of parthenogens (up to more than 600 in some colonies) may dramatically boost the colony's growth rate, despite the smaller size of the neotenic females, while representing a 'life insurance' against an accidental death of the queen. Moreover, because the parthenogenetic queens themselves can produce subsequent generations of female parthenogens that replace them, the founding queen conserves full genetic input long after her death. She enjoys virtual 'genetic immortality', limited only by the lifespan of the colony as a whole (Matsuura *et al.* 2009; Matsuura 2011, 2017). In long-lived species, AQS may have additional impacts on colony genetic structure, resulting from an eventual replacement of the founding primary king upon his death by a sexually produced neotenic one, which carries genes of the primary king and queen in equal proportions. As this neotenic king mates with parthenogenetically produced neotenic females, their progeny will carry the founding queen's and king's genes in a 3:1 ratio. If commonplace in the population, such a bias increases the relative reproductive value of dispersing females and favours a female-biased disperser sex ratio (Kobayashi *et al.* 2013; Matsuura 2017).

Soon after this first description of AQS in termites, a similar breeding system was confirmed to occur in two other species of the genus *Reticulitermes*, that is *R. virginicus* and *R. lucifugus* (Vargo *et al.* 2012; Luchetti *et al.* 2013). Thus, AQS has been viewed for some time as a singularity restricted to a single genus of subterranean lower termites. However, the three AQS species belong to three phylogenetically and geographically distinct lineages within the species-rich genus *Reticulitermes*, suggesting an independent evolution of AQS in the three cases (Dedeine *et al.* 2016; Matsuura 2017). More importantly, we recently showed that AQS also occurs in the family Termitidae (higher termites), the most diversified and abundant termite clade. The discovery of AQS in the two unrelated neotropical species *Embiratermes neotenicus* (Syntermitinae) (Fougeyrollas *et al.* 2015) and *Cavitermes tuberosus* (Termitinae) (Fournier *et al.* 2016), phylogenetically remote from *Reticulitermes*, suggests that the actual frequency of this outstanding breeding system across the phylogenetic diversity of Isoptera may be much higher than previously estimated (Matsuura 2017).

The independent origin of AQS in the three genera becomes even more evident when we consider the cytogenetic mechanisms underlying the restoration of diploidy during the formation of parthenogens. Unlike in *Reticulitermes*, in which automixis with terminal fusion gives rise to practically full homozygotes for one of the maternal alleles at each locus, ploidy restoration in *E. neotenicus* is automixis with central fusion, leading to the conservation of most heterozygous maternal allelic combinations in the parthenogens (Fougeyrollas *et al.* 2015). By contrast, the queen parthenogens in *C. tuberosus* are perfectly homozygous at all loci, suggesting yet another mechanism of ploidy restoration, gamete duplication (Fournier *et al.* 2016).

The currently known cases of AQS prompt questions about its adaptive role and selection forces driving its multiple independent evolution in phylogenetically and ecologically distant taxa. How the combination of

thelytoky with sexual reproduction shapes the life history of these species remains poorly understood. AQS may take place at different stages of the colony life cycle: it occurs systematically and rather early in the colony's life in *R. speratus* and *E. neotenicus* (Matsuura 2011; Fougeyrollas *et al.* 2015), but often as late as in mature colonies in *C. tuberosus* (Fournier *et al.* 2016). AQS benefits are thus likely to differ among species, but detailed insights into the reproductive structure and dynamics of the presently known AQS species are hampered by the large population and nest sizes of their colonies and by difficulties in finding young colonies.

During our survey of breeding systems in higher termites of French Guiana, we identified *Silvestritermes minutus* (Emerson, 1925) (Syntermitinae) as another candidate for AQS based on the frequent presence of multiple female nymphoid neotenics accompanied by a single primary king. Epigeous nesting in small and well-delimited nests and very high local abundances allow the collection of sufficient numbers of entire colonies at various stages of their development and a complete census of their inhabitants. Therefore, we selected *S. minutus* as a suitable model enabling us to obtain a complex image of the life history and reproductive strategy. We describe *S. minutus* as a new case of AQS, report on the breeding system and genetic structure of colonies using eight newly developed microsatellite markers and four markers used in our previous studies, and reconstruct the life cycle of the colonies and dynamics of the queen replacement. We also test for a possible female-biased allocation into alate dispersers, as predicted under AQS with common king replacement (Kobayashi *et al.* 2013). Ultimately, we compare the life histories of currently known AQS species, highlight how *S. minutus* uses the benefits of AQS for its reproductive success and propose the main adaptive roles of AQS in this species.

Material and methods

Origin of colonies and sampling

Altogether 137 colonies/nests were inspected and sampled during five missions in 2014–2016 at 13 sites along the Route to Petit Saut and by the Sinnamary river in the vicinity of the Petit Saut dam, French Guiana (N5°02.662′–N5°07.202′, W53°03.295′–W52°57.878′). The distance between individual collection sites ranged from 300 m to 11.3 km (Fig. S1, Supporting information). *Silvestritermes minutus* builds small, usually spherical or ellipsoid epigeous nests from soil material, situated most often a few centimetres above the ground level on young sprouts of the locally abundant *Astrocaryum* spp. palm trees (Arecaceae), that is *A. gynacanthum*, *A. sciophilum* and *A. vulgare* (Funk *et al.* 2007). The nests are penetrated with a network of roots, reinforcing the soft building material. In the central part, a hardened discoid royal chamber devoid of roots (Fig. S2, Supporting information) can be distinguished, containing the reproductives.

Life cycle reconstruction

The colony life cycle was reconstructed by combining the data on the social composition of colonies and nest sizes. The sampling was performed during three consecutive years and two different seasons, that is April–June and October–November, referred to below as *wet season* and *dry season*, respectively. One hundred and thirty-seven entire nests were collected, all reproductives, nymphs and alates were scored, and retrieved according to their sex and developmental stage. The queens were classified into four categories based on their maturity level, referred to below as follows: A, *nonphysogastric queen*, showing no increase in abdomen when compared to alate female disperser or freshly moulted neotenic female; B, *maturing queen*, showing slight or moderate physogastry and light pigmentation of abdominal intersegmental membrane and fat body; C, *fully physogastric queen*, reaching a maximum level of abdomen inflation when compared to other stages; D, *ageing* or *senescent queen*, with abdominal body wall shrunken and dark pigmentation of intersegmental membrane and fat body. The four maturity stages of neotenic queens are depicted in Fig. S3 (Supporting information). Reproductives, nymphs and alates were preserved in 96% or 80% ethanol for genetic analyses or anatomic observations, respectively, together with subsamples of workers and soldiers. Whenever it was possible, the nest sizes were estimated from the total volume (85 nests), calculated using the formula $l \times w \times h \times \pi/6$ for spheroid objects, where l, w and h stand for length, width and height, respectively.

Developmental origin of neotenic queens

Developmental origin of neotenic queens was studied using morphometric analysis and by direct observations of female nymphs moulting into neotenics in some of the inspected colonies. In addition, we separated 1–5 female nymphs of the fourth stage together with 20 workers and five soldiers from 10 nondispersing colonies into Petri dishes lined with moistened filter paper and observed their eventual moulting for 60 h. Nymphal stages (second to fifth), neotenic queens and alates from four colonies (126 individuals) were photographed using Olympus SZH10 stereoscope + Canon D600 camera, and following structures were measured in IMAGEJ 1.48: head width, right fore wing bud or wing length

from apex to anterior margin, left hind tibia length, width and length of pronotum and length of the growth zone of the antenna from the basis of the third segment to the apex of the ninth segment counted from the antennal tip. To correct for possible size differences among colonies, the data were centralized for the head width of the fourth-stage female nymphs. The data were visualized using principal component analysis in STATISTICA 8.

Development of microsatellite markers

Total genomic DNA was isolated from heads and thoraces of eight pooled samples of five *S. minutus* soldiers from two colonies, following the DNeasy® Blood & Tissue Kit (Qiagen, France) protocol with a final elution in 50 μL of buffer. One milligram was used for the production of microsatellite libraries by GenoScreen (France) through 454 GS-FLX titanium pyrosequencing as described in Malausa *et al.* (2011). A total of 4933 sequences comprising a microsatellite array and 94 pairs of flanking primers were identified in silico. Eight primer pairs were biologically validated with respect to successful amplification of microsatellite sequences and desired polymorphism of the corresponding microsatellite arrays in biological samples.

Microsatellite characterization was performed using one soldier from 74 colonies collected throughout Petit Saut area, hereafter referred to as 'population data set'. Individuals were extracted as described above and genotyped for eight *S. minutus* loci de novo developed for this study, for three loci developed previously for *Embiratermes neotenicus*, that is En-15 (Fougeyrollas *et al.* 2015), En-35 and En-39 (R. Fougeyrollas, K. Dolejšová, J. Křivánek, D. Sillam-Dussès, R. Hanus, V. Roy, in preparations), and for one locus developed for *Labiotermes labralis*, that is Lal-5 (Dupont *et al.* 2009). PCRs were performed in a total volume of 12.5 μL containing 1× Qiagen Multiplex PCR Master Mix, 0.2 μM of each forward and reverse primer, 1 μL template DNA and PCR-grade water (q.s.). Following cycling conditions were used as follows: an initial denaturation step at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, an annealing step at 60°C for 90 s and an extension step at 72°C for 30 s, and a final extension step at 68°C for 10 min. Genotyping was performed using an ABI PRISM® Genetic Analyzer (Applied Biosystems, genomic platform of IMRB, Mondor Institute, France). Fragment lengths were manually evaluated on chromatograms to detect inconsistencies, and genotypes were scored against the GeneScan-500 Liz® Size Standard (Applied Biosystems) using GENEMAPPER 5 (Applied Biosystems).

The population data set was used to estimate basic population statistics. The number of alleles, expected and observed heterozygosities (H_E and H_O, respectively) and the fixation index (F_{IS}, Weir & Cockerham 1984) were calculated using GENETIX 4.05.2 (Belkhir *et al.* 2004). GENEPOP on the Web (Raymond & Rousset 1995) and FSTAT 2.9.3.2 (Goudet 2001) were used to test the deviations from Hardy–Weinberg equilibrium (HWE) with a sequential Bonferroni correction for multiple tests. Linkage disequilibrium between each pair of loci was tested using log-likelihood ratio statistics with GENEPOP on the Web. Large allelic dropouts, scoring errors due to stuttering, and null alleles were determined using MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004).

Breeding system analysis

Eight nondispersing colonies headed by the primary king and one or two generations of neotenic females (colonies A–D, F and H–J, Table 1), and two colonies containing the primary king, the primary queen and young neotenic females (E and G, Table 1), were selected for detailed analysis of breeding system with emphasis on the genetic origin of the neotenic females. The primary reproductives, 2–24 neotenic females, up to 10 female nymphs of the fourth stage, 15 workers (29 for the colony G where soldiers were not available) and 15 soldiers from each colony, were analysed. In addition, 18 workers, 18 soldiers, 30 male and 30 female alate imagoes were genotyped in two dispersing colonies (K and L, Table 1) to determine the genetic origin of alate dispersers. Total genomic DNA was extracted individually, and all 657 individuals were genotyped at the 12 validated microsatellite loci. Three PCR multiplexes were designed, and PCRs were run using protocols and cycling conditions described above, with 0.2 mM of each primer mix (Table S1, Supporting information).

Parental genotypes were reconstructed for each colony from the genotypes of workers and soldiers. Inferred parental genotypes were compared with genotypes of available primary reproductives and then assigned to maternal and paternal origin. Allelic number and distribution were scored in the genotypes of neotenic females, fourth-stage female nymphs and alates with respect to the presence of paternal alleles in order to test the sexual or parthenogenetic origin of these castes. The relatedness between the different castes was estimated using RELATEDNESS 5.0.8. (Goodnight & Queller 1998). Estimates (Queller and Goodnight's *r*) were bias corrected. Standard errors and 95% confidence intervals were calculated by jackknifing over loci. Values whose confidence interval do not overlap the expected value were considered significant with at $\alpha = 0.05$ level.

Table 1 List of colonies sampled for the genetic study on the breeding system and number of individuals genotyped

Code	Site	GPS coordinates	Collection date	Primary queen	Primary king	Alates (♀/♂)	Neotenic ♀ 1st/2nd generation	Fourth stage ♀ nymphs	Workers	Soldiers
A	P305	N5°05.320 W52°57.869	4.14	—	1	—	10/0	—	15	15
B	River	N5°04.121 W53°03.230	4.14	—	1	—	14/0	—	15	15
C	River	N5°04.131 W53°03.197	4.14	—	1	—	18/0	—	15	15
D	River	N5°04.131 W53°03.198	4.14	—	1	—	18/0	—	15	15
E	Maman Lézard	N5°04.005 W52°59.813	4.14	1	1	—	2/0	—	15	15
F	Clio	N5°06.054 W52°57.896	4.14	—	1	—	18/0	—	15	15
G	RR1	N5°04.430 W52°58.753	10.14	1	1	—	10/0	—	29	—
H	RR1	N5°04.342 W52°58.735	4.15	—	1	—	20/4	3	15	15
I	RR1	N5°04.257 W52°58.767	4.15	—	1	—	10/1	10	15	15
J	Football field	N5°04.421 W53°03.243	4.15	—	1	—	16/0	—	15	15
K	River	N5°04.104 W53°03.214	6.16	—	—	30/30	—	—	18	18
L	River	N5°04.098 W53°03.261	6.16	—	—	30/30	—	—	18	18

The cytogenetic mechanism of parthenogenesis was determined by calculating the rates of transition to homozygosity in the first generation of parthenogenetic neotenic queens for the loci heterozygous in their inferred (or genotyped) mothers, primary queens. These values were then compared to those expected under different modes of thelytoky by means of a chi-square test (Pearcy *et al*. 2006).

Sex ratio of dispersing reproductives and sex allocation

Sex ratio of alate reproductives was calculated for 14 complete dispersing colonies, containing alates, last and penultimate nymphal stages, collected during the wet season prior to dispersal flights. All alates and nymphs were collected from the colonies and retrieved with respect to their sex. First, we calculated the mean numerical sex ratio for the 14 colonies as proportion of females relative to all future dispersers in the colony. Second, the numerical sex ratio was corrected for the dry weight differences between female and male alates so as to represent the investment sex ratio. Dry weights were calculated for 20 ready-to-fly alates of each sex from five colonies, dehydrated using an ethanol series and acetone (16 h), dried in a CentriVap Vacuum Concentrator (Labconco) for 2 h and weighed using Sartorius 4501 Micro balance. The female:male dry weight ratio was found to be very stable across the five colonies (average of five colonial ratios = 1.148, SD = 0.0075, 95% CI = 1.141–1.154), and thus it was used as a female investment coefficient to convert the numerical into investment sex ratios in all colonies. Both the numerical and investment sex ratios were compared with the value 0.5 expected under equal investments into each sex by means of one-sample *t*-tests. Third, the fact of having complete colonies with practically all future dispersers allowed us to calculate the population numerical and investment sex ratios as described in Bourke & Franks (1995) so as to consider a potential bias in sex-specific investment among colonies related to their productivity.

Results

Social composition and colony life cycle reconstruction

Summary data on social composition and nest structure of 137 colonies are given in Table 2, detailed list is provided in Table S2 (Supporting information). When evaluated separately within each sampling season, the nests

could be classified into several discrete categories, described below.

In the wet season, three exclusive categories were distinguished. First, *advanced primary colonies*, headed by the primary king and a fully physogastric primary queen. In 11 of 18 cases, second- to fourth-stage female nymphs (up to 13) were observed, likely destined to become neotenic queens; in three cases, one or two nonphysogastric neotenic females were already present (Fig. 1B, Table 2). Second, *advanced secondary colonies*, containing one primary king, up to 28 maturing or fully physogastric neotenic queens, in some cases also nonphysogastric female neotenics or third- and fourth-stage nymphs (Fig. 1D, Table 2). In most colonies, the queens showed a maximum physogastry (up to 17 mg of dry weight), exceeding that of primary queens (5 mg in maximum). Third, *dispersing colonies*, containing hundreds to thousands of fourth- and fifth-stage nymphs and alates of both sexes (up to 4837) (Fig. 1F, Table 2). Most colonies were devoid of reproductives and contained only sterile castes, only rarely young brood. Only in four of 22 colonies, neotenic queens of various maturity levels were observed, with a primary (two cases) or neotenic king (one case) or without a male. External nest shell was irregular, poorly structured and densely inhabited by alates. Nest interior was restructured as well and the former royal chamber indistinct or missing.

Four mutually exclusive nest categories were distinguished in the dry season, seemingly preceding or following the three categories described above. First, very small *incipient primary colonies*, headed by a pair of primaries, with nonphysogastric or only slightly physogastric queen (Fig. 1A, Table 2). Second, *early secondary colonies*, containing a primary king and up to 21 nonphysogastric or maturing neotenic queens, often also third- and fourth-stage female nymphs (Fig. 1C, Table 2). In two of 14 cases, the primary queen was present, one of them physogastric and still egg-laying, the other senescent, with shrunken abdomen and atrophied ovaries (Fig. S4, Supporting information). In one case, the primary king was replaced by a neotenic. Third, *late secondary colonies*, headed by the primary king and up to 25 neotenic queens of various maturity levels, some queens in each colony being fully physogastric or senescent. In eight of the 14 colonies, two generations of queens with markedly different maturity levels could be distinguished (Fig. 1E, Tables 2 and S2, Supporting information). And fourth, *postdispersal nests* with restructured external shell, swarming outlets and/or alate wings. Most of the 30 nests contained only a small population of sterile castes without eggs and brood, and 11 of them were abandoned. Only in three nests reproductives were observed, that is six senescent neotenic queens with a primary king (one colony) or one neotenic queen without a male.

When the nest categories were ranked according to seasons and along increasing maturity stages of queens (Table 2), following main conclusions could be made on the colony life cycle. Colonies are founded by a pair of dispersers in late wet season and occur as incipient colonies in the next dry season. The founding queen disappears during the second year and is replaced by neotenic queens; fully physogastric primary queens are abundant in the wet season, whereas only one fertile and one senescent primary queen were observed in the dry season, both in the company of neotenic females. By the end of the second year, the first-generation neotenic queens reach a maximum fecundity but do not produce yet alate dispersers. Instead, they are eventually complemented or replaced by a second generation and some of them proceed to senescent stage. Only then the colonies reproduce by dispersal at the end of the third year, most of them being devoid of any reproductives, and soon afterwards decline. Only two replacement neotenic kings were observed, suggesting that male neotenics rarely intervene in the reproduction. Despite the large variations in nest sizes within categories, the proposed succession of life cycle stages is corroborated by increasing median nest volumes as shown in Table 2.

Developmental origin of neotenic queens

In three inspected colonies, we directly observed six female nymphs of the fourth stage, recognized by the size and shape of wing buds on the shed cuticle, during the moult into neotenic queens with characteristic shortened wing buds. Accordingly, in four of the 10 groups of separated fourth-stage female nymphs extracted from nonswarming colonies, 1–5 nymphs moulted into neotenics within 60 h. Within the next 6–12 h, the newly moulted neotenic females acquired the characteristic pigmentation of the head and abdominal sclerites (Fig. S3, Supporting information). The origin of neotenic queens from fourth-stage nymphs was independently confirmed by the morphometric analysis, indicating that by general morphological parameters the neotenic queens are equivalent to fifth-stage nymphs, except for the reduced wing buds (Fig. S5, Supporting information).

Microsatellite characteristics

The 12 microsatellite loci showed a number of alleles ranging from 2 (Sm-02) to 20 (Sm-16). No significant deviation from HWE was observed for any of the loci ($P > 0.05$) and none of the 66 pairs of loci was in

Table 2 Social structures of 137 colonies collected during three consecutive years in two seasons (WET and DRY)

		Social structure category						
Castes and stages present/nest characteristics	Queen maturity level	Incipient colony	Advanced primary colony	Early secondary colony	Advanced secondary colony	Late secondary colony	Dispersing colony	Postdispersal nest (colony)
Primary king		2	18	13	37	14	2	1
Neotenic king				1			1	
Primary queen	A	1						
	B	1						
	C		18	1				
	D			1				
Neotenic queen(s)	A		3 (1, 1–2)	8 (3, 2–18)	8 (1, 1–4)	3 (3, 3)	2 (2, 1–3)	1 (1)
(median number per colony, range)	B			7 (13, 11–21)	8 (4, 1–11)	5 (2, 1–9)	1 (13)	1 (1)
	C				31 (8, 3–26)	6 (7, 3–22)	2 (15, 5–25)	
	D					8 (7, 2–16)	1 (7)	1 (6)
Nymphs (♂ << ♀ < 100)			11	10	18	11		11
Workers, soldiers		2	18	14	37	14	22	19
Brood, eggs		2	18	14	37	14	3	
Alates, late nymphs (♂:♀ ≈ 1:1 >> 100)							22	
Symptoms of past dispersal								30
Dry season		**2**		**14**		**14**		**30**
Wet season			**18**		**37**		**22**	
Nest size (cm^3) median		73	308	907	1713	2746	7263	10 808
quartiles		63–84	245–592	737–1118	1182–2863	1760–424	2224–12 374	3486–16 318
(*n*)		(2)	(11)	(12)	(19)	(11)	(20)	(10)

Total numbers of colonies from each social category in each season are given in bold. Numbers indicate the numbers of colonies in which individual castes and stages or nest characteristics were observed. Letters indicate the levels of queen maturity: A, young, nonphysogastric; B, maturing, slight to advanced physogastry; C, fully physogastric; D, ageing or senescent, abdominal cuticle dark and shrunken. Symptoms of past dispersal include restructured external nest shell, presence of dispersal outlets and alate wings.

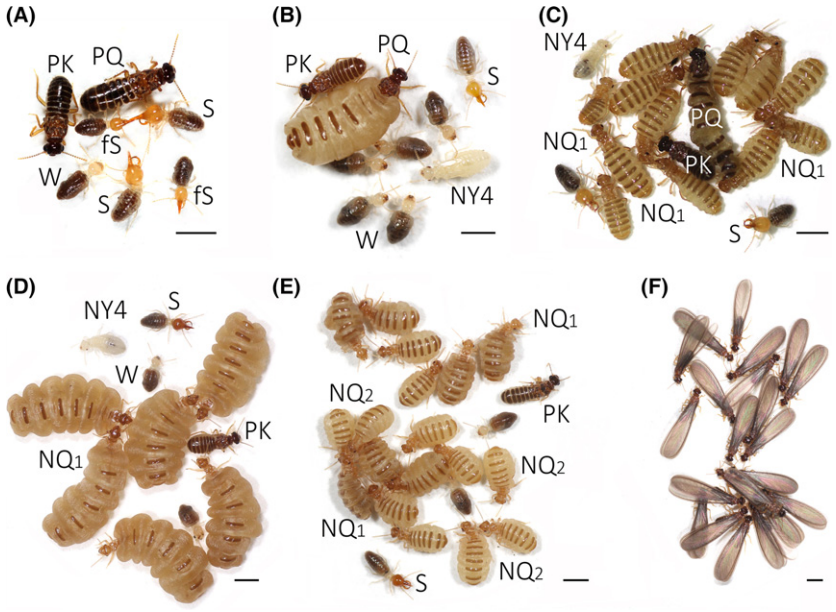

Fig. 1 Social structures of colonies in different stages of the life cycle based on observations of 137 colonies collected over three successive years in two different periods of the year. (A) Incipient primary colony ca. 4 months after colony foundation. (B) Advanced primary colony ca. 11 months after colony foundation. (C) Early secondary colony during the replacement period, with the primary queen still present, but senescent and with atrophied ovaries, ca. 16 months after colony foundation. (D) Advanced secondary colony with fully physogastric neotenic queens of the first generation, ca. 22 months of the colony life. (E) Late secondary colony, with ageing neotenic queens being gradually replaced by the second generation of neotenic females, ca. 28 months of the colony existence. (F) Dispersing colony containing numerous alates of both sexes and sterile castes, only rarely neotenic queens and primary king, 3 years after colony foundation. PK, primary king; PQ, primary queen; NQ_1, first generation neotenic queens; NQ_2, second generation neotenic queens; NY4, fourth-stage female nymph; S, soldier; fS, first soldier; W, worker. Scale bars represent 2 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

significant linkage disequilibrium ($P > 0.01$). Mean observed and expected heterozygosities were 0.712 (SD = 0.262) and 0.732 (SD = 0.267), respectively. No evidence of null alleles, large allelic drop-out or stutter bands was detected. Microsatellite characteristics are summarized in Table S1 (Supporting information).

Breeding system analysis

Summary of genotypes recorded in the 657 analysed individuals is given in Table S3 (Supporting information), the complete list of genotypes was deposited in Dryad as https://doi.org/10.5061/dryad.s056d. Parental genotype reconstruction identified a single pair of founding reproductives in each of the 12 colonies studied (A–L, Table S3, Supporting information), and genotypes of sterile castes did not show any significant deviation from Mendelian distribution ($P = 0.2907$–0.9591). Observed genotypes of sampled primary kings and queens, when present, always matched with inferred parental genotypes. The level of relatedness (r) was equal to 0.5237 (SE = 0.0135, 95% CI = 0.4939–0.5535) among workers and soldiers, to 0.5052 (SE = 0.0260, 95% CI = 0.4479–0.5625) between workers/soldiers and the primary king, to 0.5253 (SE = 0.0235, 95% CI = 0.4736–0.5770) between workers/soldiers and the inferred primary queen, and to 0.4131 (SE = 0.0257, 95% CI = 0.3566–0.4696) between workers/soldiers and neotenic females. Only the latter value was slightly but significantly different from 0.5 ($P < 0.05$).

A total of 141 neotenic females, including five females of the second generation and 13 female nymphs of the fourth stage collected from nondispersing colonies, were genotyped. For 134 neotenic females, including four of the second generation, and all 13 female nymphs, a maximum of two alleles and three genotypes was observed per colony and locus (Table S3, Supporting information). Genotypes of these females were incompatible with sexual reproduction. They had only inferred/observed maternal alleles, while exclusive paternal alleles were never observed. When the mother was heterozygous, neotenic females were either heterozygous or homozygous for one of the maternal alleles at the given locus. They were strongly related to the primary queen ($r = 0.7959$, SE = 0.0292, 95% CI = 0.7317–0.8601) but unrelated to the primary king ($r = 0.0181$, SE = 0.0415, 95% CI = −0.0733 to 0.1095). Thus, most of the neotenic females and all fourth-stage female nymphs were

produced by thelytokous parthenogenesis. For the remaining seven neotenic females, including one neotenic female of the second generation, one of the two alleles of the primary king was observed at all loci, providing evidence for their sexual origin. The relatedness value between these neotenic females and the primary queen (r = 0.5926, SE = 0.0487, 95% CI = 0.4854–0.6998) and primary king (r = 0.4004, SE = 0.0970, 95% CI = 0.1870–0.6138) did not significantly deviate from 0.5 ($P < 0.05$). Thus, this small proportion (5%) of neotenic females was sexually produced. Proportions of parthenogenetically and sexually produced individuals of all castes are summarized in Fig. 2.

We used the model predictions of Pearcy *et al.* (2006) to estimate the mode of ploidy restoration during the parthenogenetic process. Locus En-39 was discarded because only two primary queens were heterozygous at this locus. At all other loci, three to 10 primary queens were heterozygous and produced 42–132 heterozygous neotenic females of the first generation (Table 3). The rate of transition to homozygosity (R) ranged from 5% to 34% depending on the locus, and all values were significantly different from those expected under apomixis and gamete duplication models. For four loci, the R values were not significantly different from those expected under automictic thelytoky with terminal fusion, central fusion and random fusion. For the seven remaining loci, R values were not significantly different only from those expected under automixis with central fusion.

A total of 120 alates were genotyped in colonies K and L. All 60 males and most of the females (56) contained in their genotypes the alleles of both inferred colony founders, suggesting they were produced sexually. The remaining four female alates possessed alleles of only one of the inferred parents, indicating their parthenogenetic origin (Table S3, Supporting information, Fig. 2).

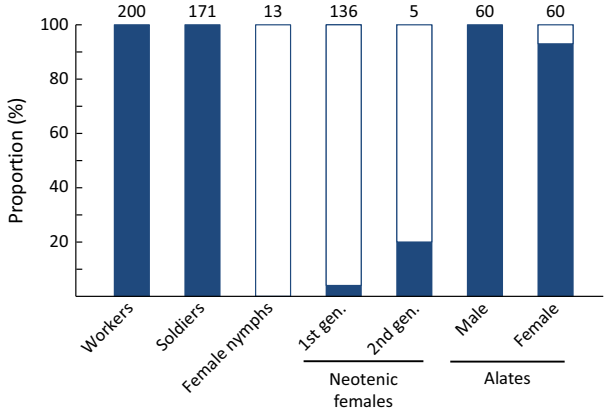

Fig. 2 Proportions of sexually (filled bars) and parthenogenetically (open bars) produced individuals in the 12 genotyped colonies, that is workers, soldiers, fourth-stage female nymphs from nondispersing colonies, two generations of neotenic females, and alate dispersers of both sexes. Numbers above each bar represent the total number of individuals genotyped. [Colour figure can be viewed at wileyonlinelibrary.com]

Sex ratio of dispersing reproductives and sex allocation

We collected all alates, last or penultimate stage nymphs (23 403 individuals) in 14 dispersing colonies in the wet seasons 2015 and 2016, with the mean of 1672 (SD = 1215) and a maximum of 4837 future dispersers per colony (Table S4, Supporting information). The average numerical colonial sex ratio was slightly, though, not significantly male-biased (mean = 0.480, SD = 0.054, $t = -1.402$, $P = 0.18$), and the colonial investment sex ratio was very close and not significantly different from the value expected under equal sex investment (mean = 0.514, SD = 0.054, $t = 0.93$, $P = 0.366$) (Fig. 3). At the population level, the numerical sex ratio was calculated to be slightly but significantly male-biased (mean = 0.470, 95% CI = 0.444–0.496), while the population investment sex ratio revealed to be equal (0.504, 95% CI = 0.478–0.531) (Fig. 3, Table S4, Supporting information).

Discussion

In this study, we identify *Silvestritermes minutus* as a new species of higher termites adopting the outstanding reproductive strategy called AQS. We show that the colonies are established by a pair of outbred primary reproductives and that the founding queens are replaced at an early stage of the colony life cycle (as early as during the second year) by relatively low numbers of highly fecund neotenic queens. These develop from fourth-stage nymphs, mostly arising from unfertilized eggs through automictic thelytokous parthenogenesis with central fusion. By contrast, workers and soldiers are produced by a conventional sexual process. After the primary queen replacement, the neotenic queens reproduce with the primary king and are eventually complemented by a new generation of their own parthenogens. The colony reproduction is most often restricted to a single large dispersal of male and female alates, mostly produced sexually and in unbiased sex ratios, 3 years after colony establishment. Soon afterwards, most colonies decline and disappear.

Silvestritermes minutus adds to the list of higher termites with AQS, together with the first two cases that were recently described in two other neotropical species, *Embiratermes neotenicus* (Fougeyrollas *et al.* 2015) and *Cavitermes tuberosus* (Fournier *et al.* 2016). The

Table 3 Transitions to homozygosity in parthenogenetic neotenic queens of the first generation

Locus	PQ_{het}	NF_{tot} from PQ_{het}	NF_{hom}	*R*	Apomixis (*r* = 0)	Automixis			
						Gamete duplication (*r* = 1)	Terminal fusion (*r* = 0.33–1)	Central fusion (*r* = 0–0.33)	Random fusion (*r* = 0.33)
Sm-02	3	42	3	0.07	***	***	***	NS	***
Sm-05	7	96	33	0.34	***	***	NS	NS	NS
Sm-22	8	96	18	0.19	***	***	**	NS	**
Sm-25	10	130	19	0.15	***	***	***	NS	***
Lal-05	9	117	6	0.05	***	***	***	NS	***
En-35	7	89	25	0.28	***	***	NS	NS	NS
Sm-06	8	118	11	0.09	***	***	***	NS	***
Sm-16	10	132	36	0.27	***	***	NS	NS	NS
Sm-23	9	116	30	0.26	***	***	**	NS	**
Sm-27	9	115	35	0.30	***	***	NS	NS	NS
En-15	9	117	22	0.19	***	***	**	NS	**

PQ_{het}, number of heterozygous inferred primary queens; NF_{tot}, total number of the first generation parthenogenetic female neotenics from a heterozygous mother; NF_{hom}, number of homozygous neotenic females; *R*, observed rate of transition to homozygosity; *r*, expected generational rate of transition to homozygosity; NS, not significant.
***$P < 0.001$.
**$P < 0.01$.

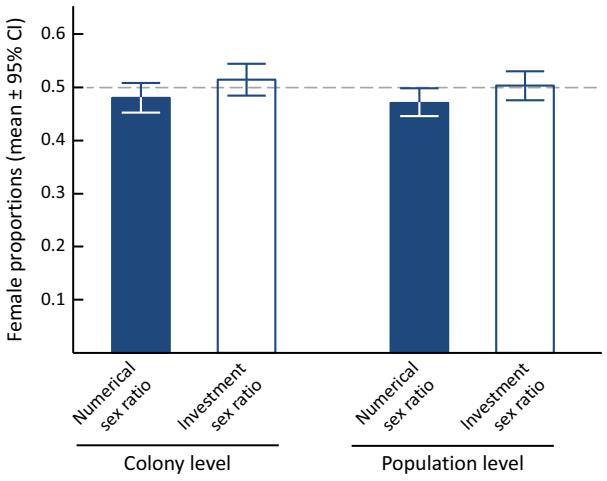

Fig. 3 Sex ratio of future alate dispersers calculated for 23 403 late stage nymphs and alates originating in 14 dispersing colonies, expressed as proportion of females. Filled bars show the numerical sex ratio, open bars show energetic investment into females, calculated as unweighted mean proportion in the 14 colonies (left) and as population averages, taking into account the total productivity of each colony (right). [Colour figure can be viewed at wileyonlinelibrary.com]

genus *Silvestritermes* is phylogenetically remote from *Cavitermes*, each of them belonging to another subfamily of Termitidae, as well as from *Embiratermes* within Syntermitinae (Rocha *et al.* 2012; Kyjaková *et al.* 2017). Therefore, it likely represents another case of independent AQS evolution in higher termites, in addition to the multiple occurrences in the lower termite genus *Reticulitermes* (Dedeine *et al.* 2016). The polyphyletic origin of AQS is reflected also in the diversity of cytogenetic mechanisms of the parthenogenetic process in different lineages. Automixis with terminal fusion described in *Reticulitermes* (Matsuura *et al.* 2009; Vargo *et al.* 2012; Luchetti *et al.* 2013) and gamete duplication observed in *C. tuberosus* (Fournier *et al.* 2016) are complemented by automixis with central fusion in *E. neotenicus* (Fougeyrollas *et al.* 2015). Our data for *S. minutus* provide a robust support to automixis with central fusion as well. The rate of transition to homozygosity during the formation of queen parthenogens was consistent with theoretical predictions for central fusion at all 12 analysed loci, the variability among individual loci (5–34%) likely reflecting different probabilities of recombination due to different positions on the chromosomes (Pearcy *et al.* 2006). Thus, the mode of ploidy restoration in *S. minutus* is identical with the one proposed for the phylogenetically closest AQS species known, to date, *E. neotenicus*.

The description of the new case of AQS in *S. minutus* once again invokes the question on the real incidence of this reproductive strategy in Isoptera. The currently known cases do not provide a clear image of determinants promoting the evolution of AQS. On the one hand, there is no intelligible clue in the diversified life histories, feeding habits and ecology of these species, on the other hand, for each AQS species several related, sympatric and ecologically close species can be listed that are lacking AQS. In the case of *S. minutus*, the congeneric species *S. heyeri* is a widespread South

American species living in sympatry with *S. minutus* over large areas. Both species are humivorous, *S. heyeri* being of larger body size and building larger nests, usually situated several decimetres above ground on the trunks of grown trees. A careful inspection of numerous *S. heyeri* nest did not provide any indices of queen replacement in this species, and even large nests were always headed by a single primary king and one highly physogastric primary queen (R. Hanus, J. Křivánek, K. Dolejšová, personal observation).

Along with the multitude of independent origins and different mechanisms underlying the parthenogenetic process, the adaptive significance of AQS also appears to differ among individual cases. In *S. minutus*, the replacement of the foundress by neotenic parthenogens represents an obligatory event in the life cycle of the colony. Already 1 year after colony establishment, the first female nymphs destined to replace the queen are present, and sometimes the primary queen is already accompanied by the first neotenic females. While in the middle of the second year, two cases of functional or senescent primary queens were still observed, all 2-year-old colonies contained physogastric neotenic females and a primary king. Thus, the role of the primary queen is restricted to colony foundation and rapid production of the first generation of neotenic queens. Colonies containing the primary queen were never observed to produce alate dispersers. The latter are exclusively produced by the primary king with a harem of neotenic queens, and the dispersal takes place by the end of the third year, almost 2 years after the primary queen replacement. Most dispersing colonies were already devoid of reproductives and young brood, suggesting their decline after the dispersal flights. Accordingly, most postdispersal nests were abandoned or inhabited by sterile castes; only rarely these colonies contained neotenic females, and only one of them also the primary king.

The adaptive role of AQS in *S. minutus* seems to be the maximization of allocation into a single dispersal event within a very short life cycle through the replacement of one queen by multiple parthenogens. Their relatively low number (maximum of 32 neotenic females of two generations) in comparison with other AQS species is compensated by their great physogastry, corresponding to a dry weight reaching more than three times that of the primary queens. AQS in *S. minutus* can thus be viewed as an essential element of the species' life history strategy; it allows fast colony growth and rapid release of fertile dispersers, compensating for probable vulnerability to environmental pressures due to small body size and small nests from soft building material situated very close to the ground. In other words, *S. minutus* benefits from AQS to boost its population as fast as possible rather than to extend the colony lifespan. This contrasts with the limited knowledge on other AQS species. Our observations on *E. neotenicus* suggest that the primary queen is also replaced very early (Fougeyrollas *et al.* 2015), but its large and robust nests with several hundred neotenic queens and one primary king persist 4 or more years and regularly release dispersing alates (R. Hanus, J. Křivánek, K. Dolejšová, personal observation). In *C. tuberosus*, the founding queen may survive several years and is often still active when alate dispersers are produced. Its replacement by the harem of parthenogens is thus a rather late and facultative event, allowing the colony to extend its lifespan once the colony successfully reproduced (Fournier *et al.* 2016). In sum, while the general benefits offered by AQS, that is succession of queen generations with undiluted genetic input of the foundress, multiplication of reproductive potential of the colony and prevention of inbreeding in sterile castes and dispersers are theoretically available to all species with AQS syndrome, each of them uses these benefits at different rates and in species-specific combinations.

The short lifespan of *S. minutus* colonies brings along other consequences for the colony genetics. Within the 3 years of the colony life, neotenic kings only very rarely replace the primary kings. We observed only two cases of king replacement during the survey of colony social composition. Under AQS, a systematic replacement of founding primary kings by their sons, mating with the primary queens' parthenogens, has been proposed to be the driving force for female-biased sex ratio in dispersing alates due to kin selection for alleles of female origin, being more likely to be transmitted by alates to future generations (Matsuura 2011; Kobayashi *et al.* 2013). This prediction has been experimentally confirmed in two species of *Reticulitermes* with AQS; a significantly higher investment into female alates has been found to correlate with the estimated frequencies of king replacement in the two species, while being absent in congeneric species lacking the AQS breeding system (Kobayashi *et al.* 2013). By contrast, our observations in *S. minutus* do not show any asymmetries in sex allocation into alate dispersers. While the numerical sex ratio was slightly male-biased, the investment sex ratio, calculated from more than 23 000 future dispersers from 14 colonies, was unbiased. In fact, these results are in line with Kobayashi *et al.*'s (2013) hypothesis, given the rarity of king replacement by neotenic males. Thus, the very rare incidence of mother–son inbreeding is unlikely to have shaped the sex allocation. Nevertheless, the case of *S. minutus* shows that the presence of AQS cannot be predicted or excluded based on sex ratios of dispersers alone, as previously proposed

(Matsuura 2011; Vargo *et al.* 2012; Kobayashi *et al.* 2013), without considering other life history characteristics of the species.

One of interesting questions related to AQS evolution is what are the mechanisms responsible for the developmental priority of parthenogenetically produced female nymphs to develop into neotenic queens and vice versa, what prevents the sexually produced nymphs from developing into female neotenics and makes them become alates. The developmental priority was previously ascribed to a multilocus homozygous determination system in the case of *Reticulitermes*, in which the queen parthenogens are homozygous at a majority of loci unlike the sexually produced alate-destined nymphs; homozygosity at specific loci in the parthenogens has been shown to be linked with their priority to develop into neotenics (Matsuura 2011, 2017; Yamamoto & Matsuura 2012). Yet, this hypothesis is less likely to apply in the case of *E. neotenicus* and *S. minutus*, in which the parthenogens conserve most of the maternal heterozygosity due to automixis with central fusion (in average 96% and 80% per locus, respectively), while still showing an almost exclusive priority to become neotenics. Therefore, alternative mechanisms should also be considered, such as genomic imprinting, as proposed by Matsuura (2017). Whatever the mechanistic basis of developmental priority of parthenogens to become neotenics may be, the mechanism appears not to be perfect. Just as in all three *Reticulitermes* AQS species (Matsuura *et al.* 2009; Vargo *et al.* 2012; Luchetti *et al.* 2013) and *C. tuberosus* (Fournier *et al.* 2016), a small proportion of *S. minutus* neotenic females was produced sexually and, vice versa, a small portion of female alates was of parthenogenetic origin.

During our campaign, investigating the breeding systems of higher termites in a small area of rainforest in French Guiana, as many as three different AQS species of Termitidae have been identified, and several additional candidates are under investigation. In most tropical species, details on breeding systems are unknown and rather difficult to obtain due to their nesting habits. In addition, inferences made from genetic structure of sterile castes are not very useful as they are masking the true occurrence of AQS, which is not manifested in the genotypes of sterile colony members. Therefore, we can indirectly predict here that multiple cases of AQS can be expected in termites, including the higher termites, throughout the tropics and subtropics of South America as well as other continents.

Despite the great evolutionary distance between ants and termites, mixed modes of reproduction occurred multiple times independently in both clades. Of course, the fundamental differences in their developmental and life history characteristics, such as haplodiploid genetic architecture, holometabolous development and short-lived males in ants vs. long-lived kings and diplodiploid hemimetaboly, allowing the development of by definition wingless neotenic reproductives in termites, ultimately generate different breeding systems in the two clades. Yet, both groups succeeded in recruiting the thelytoky to take the best from both the sexual and asexual processes, within the limits given by their respective evolutionary constrains. Sporadic or accidental production of thelytokous eggs, for instance in emergency situations such as colony orphaning or lack of the mating partner, is reported in a number of ant and termite species (see e.g. in Rabeling & Kronauer 2013 for ants, Matsuura 2011 and Kobayashi & Miyaguni 2016 for termites). However, only a handful of cases have been reported so far in which thelytoky became a systematic or obligatory element of the reproductive strategies. These are particularly diverse in ants, sometimes bringing along other peculiarities such as loss of males or male clonality (Fournier *et al.* 2005; Ohkawara *et al.* 2006; Himler *et al.* 2009; Pearcy *et al.* 2011). In such situations, only the worker progeny arises through genetic mixing of males and queens, while the reproductives of both sexes remain permanently genetically separated. This is particularly advantageous in invasive populations, in which it prevents inbreeding depression due to sib mating, being likely in part responsible for the great colonization success of these species (Foucaud *et al.* 2010; Pearcy *et al.* 2011). At the first view, the breeding systems reported in the six species of Isoptera with AQS syndrome appear as less diversified. Yet, this apparent uniformity may mask differences in the genetic background of AQS resulting from different automictic mechanisms (terminal vs. central fusion). And, last but not least, as we show in the present study, the role of AQS in the life cycle of the species may be very diverse as well, leading to idiosyncratic life histories in different cases of AQS emergence.

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V.R., R.H., Y.R., and D.S.D. designed the study. J.K., K.D. and R.H. collected the material. J.K. and R.H. sampled all colonies and studied the lifecycle and sex allocation. R.F., V.R., S.F. and D.S.D. developed the microsatellite markers and analysed the genetics of colonies. R.H. and V.R. wrote the manuscript; all other co-authors contributed to their respective parts of the manuscript and approved its final version.

Data accessibility

Microsatellite loci newly developed for this study can be found under GenBank accession nos. KY614239–KY614249 and as Dryad entry doi: 10.5061/dryad.s056d. The complete list of genotypes recorded in the 657 analysed individuals was deposited in Dryad as the same entry.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Microsatellite loci used for the genetic study and their characteristics studied in one soldier from 74 colonies collected throughout Petit Saut area.

Table S2 List of 137 colonies of *S. minutus* used for life cycle reconstruction.

Table S3 Genotypes recorded in 12 *S. minutus* colonies studied with respect to the reproductive structure and genetic origin of castes.

Table S4 Sex ratio of all alates and late stage nymphs collected in 14 *S. minutus* colonies in the wet season prior to dispersal.

Fig. S1 Map of the Petit Saut area showing the 13 collection sites.

Fig. S2 Cross section of the royal chambers of *S. minutus* nests.

Fig. S3 Development and maturation stages of neotenic queens.

Fig. S4 Gonads dissected from reproductives found in an early secondary colony during the primary queen replacement.

Fig. S5 Developmental origin of neotenic females.