PROCEEDINGS B

rspb.royalsocietypublishing.org

Research

Cite this article: Fougeyrollas R, Dolejšová K, Sillam-Dussès D, Roy V, Poteaux C, Hanus R, Roisin Y. 2015 Asexual queen succession in the higher termite *Embiratermes neotenicus*. *Proc. R. Soc. B* **282**: 20150260.
http://dx.doi.org/10.1098/rspb.2015.0260

Received: 4 February 2015
Accepted: 30 April 2015

Subject Areas:
genetics, ecology, evolution

Keywords:
thelytokous parthenogenesis, breeding system, termites, Isoptera, Termitidae, reproductive strategies

Author for correspondence:
Robert Hanus
e-mail: robert@uochb.cas.cz

†These authors contributed equally to this study.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2015.0260 or via http://rspb.royalsocietypublishing.org.

THE ROYAL SOCIETY PUBLISHING

Asexual queen succession in the higher termite *Embiratermes neotenicus*

Romain Fougeyrollas[1,†], Klára Dolejšová[2,3,†], David Sillam-Dussès[4,5], Virginie Roy[1], Chantal Poteaux[5], Robert Hanus[2] and Yves Roisin[6]

[1]iEES-Paris, Université Paris-Est Créteil, Créteil, France
[2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[3]Faculty of Science, Charles University in Prague, Prague, Czech Republic
[4]iEES-Paris, IRD—Sorbonne Universités, UMR 242, Bondy, France
[5]LEEC, EA4443, Université Paris 13—Sorbonne Paris Cité, Villetaneuse, France
[6]Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium

Asexual queen succession (AQS), in which workers, soldiers and dispersing reproductives are produced sexually while numerous non-dispersing queens arise through thelytokous parthenogenesis, has recently been described in three species of lower termites of the genus *Reticulitermes*. Here, we show that AQS is not an oddity restricted to a single genus of lower termites, but a more widespread strategy occurring also in the most advanced termite group, the higher termites (Termitidae). We analysed the genetic structure in 10 colonies of the Neotropical higher termite *Embiratermes neotenicus* (Syntermitinae) using five newly developed polymorphic microsatellite loci. The colonies contained one primary king accompanied either by a single primary queen or by up to almost 200 neotenic queens. While the workers, the soldiers and most future dispersing reproductives were produced sexually, the non-dispersing neotenic queens originated through thelytokous parthenogenesis of the founding primary queen. Surprisingly, the mode of thelytoky observed in *E. neotenicus* is most probably automixis with central fusion, contrasting with the automixis with terminal fusion documented in *Reticulitermes*. The occurrence of AQS based on different mechanisms of ploidy restoration raises the hypothesis of an independent evolutionary origin of this unique reproductive strategy in individual lineages of lower and higher termites.

1. Introduction

Sexual reproduction is one of the greatest evolutionary inventions shaping life on the Earth. Yet, despite indisputable advantages, the sexual process has numerous drawbacks, including the costs related to mating and the dilution of an individual's genetic contribution to the next generation. Therefore, across a wide range of multicellular organisms, conditional or obligatory use of asexual reproduction complements the dominant role of sex [1,2]. Evidence accumulates that some social insects can also facultatively combine the best from both modes of reproduction [3,4]. One of the most remarkable mixed strategies alternating asexual and sexual reproduction was described a decade ago in *Cataglyphis* ants, in which the queens produce genetically diversified worker offspring sexually and new queens through thelytokous parthenogenesis [5]. In doing so, the queens maximize their own genetic contribution to the next generation of female reproductives while maintaining a desirable diversity in helpers. This discovery has prompted a number of studies confirming a similar reproductive system in several other ant species, and unravelling how the other players in ant colonies, the workers and the males, counteract the queen's genetic dominance (reviewed in [3,4]).

Despite the great evolutionary distance between eusocial hymenopterans and termites, an analogous reproductive strategy has recently been discovered in this second major group of social insects. First observed in the Japanese subterranean termite *Reticulitermes speratus* [6] and later confirmed in two other

Table 1. Castes observed and sampled in the 43 collected colonies.

	primary queen	primary king	neotenic queens	workers and soldiers	no. colonies	neotenic males (present/absent)	nymphs (present/absent)
	×	×		×	1	0/1	0/1
		×	×	×	12	6/6	11/1
			×	×	12	3/9	5/7
				×	18	0/18	3/15
no. colonies	1	13	24	43	43	9/34	19/24

species of this genus, North American *R. virginicus* [7] and European *R. lucifugus* [8], the so-called asexual queen succession (AQS) fascinates by its convergences with the similar breeding system in ants while still having specific and exclusive traits. The colonies are usually founded by a single pair of unrelated primary reproductives. However, at a certain stage of colony development, the founding primary queen produces up to several hundred non-dispersing neotenic queens through thelytokous parthenogenesis. These parthenogens develop from female nymphs, coexist for some time with their mother and mate with the present primary king to finally replace the primary queen upon her death. The other castes (i.e. workers, soldiers and winged dispersing males and females) are produced via sexual reproduction of the primary king with the present queen(s). The colony life cycle can then continue with further cycles of queen replacement by new generations of neotenic female parthenogens. The perpetuity of AQS cycles not only boosts the reproductive potential of the colonies with hundreds of queens instead of a single one, but also results in a virtual immortality of the founding primary queen since all fertile females in the colony bear only her genetic material [9]. Unlike in most thelytokous ants, which produce queen parthenogens through automixis with central fusion, the mode of thelytoky in *Reticulitermes* is automixis with terminal fusion and the parthenogenetic queens are almost completely homozygous [6–8]. Thus, though functionally analogous, AQS in ants and *Reticulitermes* termites is based on different cytological mechanisms.

The discovery of AQS in *Reticulitermes* raises the question of whether this reproductive strategy is just a singularity restricted to a few species of a single genus of lower termites or a more widespread phenomenon overlooked in the past. This question is relevant since neotenic reproductives occur in high numbers per colony in many other termite species [10]. Higher termites (Termitidae) are by far the most successful lineage of Isoptera in terms of abundance, species richness and ecological diversification. Breeding systems reported in the higher termites are diverse as well, including records of species with frequent occurrence of multiple neotenics [10]. These species are of special interest for the search for possible occurrence of AQS. In the present study, we investigated the colony structure, reproductive strategies and genetic constitution in the colonies of the Neotropical higher termite *Embiratermes neotenicus* Holmgren (Termitidae: Syntermitinae) using five newly developed polymorphic microsatellite markers. In this abundant humivorous species, multiple neotenic queens frequently accompany a single primary king [11], and thus *Embiratermes neotenicus* is a well-suited candidate for AQS in higher termites.

2. Material and methods

(a) Origin of colonies and sampling

Embiratermes neotenicus builds large epigeous nests from soil material, usually at bases of standing trees. In October 2012, May 2013 and March 2014, we collected 43 nests or nest fragments of *E. neotenicus* at 10 localities along the Petit Saut Road, French Guiana (N5°03.017′–5°07.202′, W52°57.872′–53°03.196′), separated by 0.8–11 km. The colonies were brought to the research station Hydreco near Petit Saut dam and dissected. All reproductives found (primary kings, primary queen and neotenics) were scored, separated according to their sex under a stereomicroscope and stored in 96% ethanol. Along with the reproductives, workers, soldiers and nymphs (if present) were sampled in each colony. The reproductive structure of the sampled colonies is summarized in table 1. Species identity of each colony was confirmed to be *E. neotenicus* using DNA barcoding. The standard barcode gene, cytochrome oxidase I, was sequenced from one individual belonging to each of the 43 colonies and sequences were entered in the Barcode of Life Database (BOLD) identification system (www.barcodinglife.org). Four haplotypes (see Data accessibility section) were identified, sharing 99.4, 99.68, 100 and 99.84% identity with the sequence of reference for *E. neotenicus*.

(b) Development of microsatellite markers

Total genomic DNA was extracted from the thorax and abdomen of 20 workers from colony B, after removing the digestive tube. The tissues were pooled in four tubes, each containing five thoraces and five abdomens. Extractions were performed following Qiagen DNeasy Blood & Tissue Kit protocol with 30 min of final elution in 50 µl of distilled water. The development of microsatellite markers was performed by Genoscreen (France) using next-generation 454 GS-FLX titanium pyrosequencing of microsatellite-enriched DNA libraries [12]. Primer pairs were designed for 219 sequences from the total of 12 493 sequences with microsatellite motifs, and 23 primer pairs were selected *in silico*. Nine primer pairs were both biologically validated (i.e. successfully amplified from biological samples) and identified as polymorphic by Genoscreen. Among them, one locus showed an unusual pattern of distribution of allelic frequencies dependent on caste and/or sex and another showed a very weak amplification. Both were removed from further analysis.

One soldier (one worker in colony A) from each of the 43 colonies was genotyped at the seven remaining loci to avoid bias because of relatedness among individuals from the same colony. Monoplex PCRs were performed in a total volume of 12.5 µl containing 1 U FastStart Taq DNA Polymerase (Roche Diagnostics), 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.24 mM dNTP mix, 0.4 µM of each forward and reverse primer, 1 µl (approx. 15 ng) DNA and PCR-grade water (q.s.). The following cycling conditions were used: an initial denaturing step at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s; annealing at

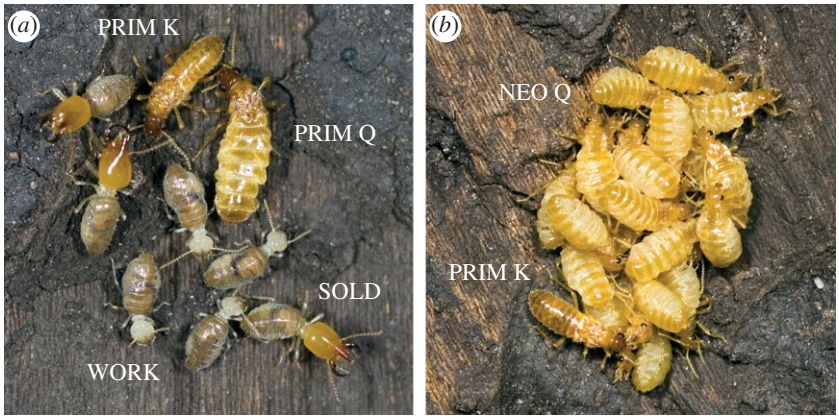

Figure 1. Photographs of *Embiratermes neotenicus* reproductives. (*a*) Primary king (PRIM K) and primary queen (PRIM Q) excavated from a small nest together with soldiers (SOLD) and workers (WORK). (*b*) Primary king (PRIM K) and numerous nymphoid neotenic queens (NEO Q) excavated from a large nest. (Online version in colour.)

55°C for 30 s; extension at 72°C for 60 s; and a final extension step at 72°C for 10 min. Genotyping was performed using an ABI PRISM Genetic Analyzer (Applied Biosystems, genomic platform of IMRB, Mondor Institute, Créteil). Fragment lengths were manually checked on chromatograms to detect inconsistencies and genotypes were scored against the GeneScan-500 Liz Size Standard (Applied Biosystems) using GENEMARKER v. 2.6.3 software (SoftGenetics, State College, PA, USA).

GENEPOP on the Web [13,14] and GENETIX v. 4.05.2 software were used to estimate the number of alleles, expected and observed heterozygosities (H_E and H_O), and the fixation index (F_{IS} [15]). Eventual occurrence of linkage disequilibrium between each pair of loci was tested using log-likelihood ratio statistics in GENEPOP on the Web. To test the deviations from Hardy–Weinberg equilibrium (HWE), we used GENEPOP on the Web and FSTAT v. 2.9.3.2 [16]. When appropriate, sequential Bonferroni correction for multiple tests was applied. Large allelic drop-outs, scoring errors because of stuttering and null alleles were determined using MICRO-CHECKER v. 2.2.3 [17].

(c) Genetic analysis

For the genetic analysis of colony structure, we selected a subset of 10 colonies: nine of them containing the primary king and neotenic queens, and the sole colony with the primary king and the primary queen (electronic supplementary material, table S1). The 10 colonies originated from six localities, and the distance between colonies ranged from 54 m to 11.3 km (electronic supplementary material, table S2). The primary king, the primary queen or 20 neotenic queens (60 in colony C), 20 workers and 20 soldiers (if available) from each of the 10 colonies were genotyped. Thirty-eight male and female nymphs of the fourth nymphal stage were genotyped from two colonies collected in May 2013 during the nymph production season (electronic supplementary material, table S1). Two PCR multiplexes were carried out using the Qiagen Multiplex PCR Plus kit in a final volume of 12.5 µl containing 1 × Qiagen Multiplex PCR Master Mix, 0.2 µM primer mix and 10–175 ng template DNA. The same amplification conditions were applied to both multiplexes: an initial denaturation step at 95°C for 5 min followed by 35 cycles at 94°C for 30 s, an annealing step at 60°C for 90 s and an extension step at 72°C for 30 s. The final extension was fixed to 68°C for 10 min.

Parental genotypes for all colonies were reconstructed from the genotypes of workers and soldiers using GERUD 2.0 [18] and COLONY v. 2.0.1.5 [19] programs and compared with the observed genotypes of available primary kings and queen. The observed genotype frequencies of workers and soldiers were then compared with the frequencies expected under Mendelian inheritance pattern of the parental reconstructed genotypes by means of a log-likelihood ratio test (G-test) summed over loci. Relatedness [20] and F_{IC} and F_{IT} inbreeding coefficients *sensu* Thorne *et al*. [21] for the sterile castes were calculated using KINGROUP v. 2 [22] and FSTAT v. 2.9.3.2 [16] and compared with the values expected under different breeding structures—that is, simple (Mendelian) families and extended families (inbred groups containing more than two functional reproductives) [23].

Number of alleles and their distribution in the genotypes were scored in neotenic queens and nymphs with regard to the presence of paternal alleles and the Mendelian distribution of the reconstructed parental genotypes in order to conclude whether the neotenic queens and nymphs were produced sexually or by means of thelytokous parthenogenesis of the queen. To determine the mode of thelytoky responsible for the origin of neotenic parthenogens, we calculated the generational rate of transition to homozygosity for the loci heterozygous in the inferred mother and compared these values with those expected under different modes of thelytoky [24].

3. Results

(a) Field observations

We found the reproductives of at least one sex in 25 of the 43 colonies (table 1); we did not find reproductives in the other colonies, probably because they were deep underground where they are very difficult to collect. In a single colony, inhabiting a small round nest of approx. 15 cm in diameter, we found the primary queen accompanied by the primary king. The remaining 24 nests were larger and contained numerous nymphoid neotenic queens (figure 1). In 12 of these 24 nests, we succeeded in finding the primary king while in the remaining 12 colonies we did not find any king. A large majority of neotenic queens were highly physogastric, egg-laying, and seemingly of the same size and pigmentation, suggesting their simultaneous origin. Their number per colony ranged from 35 to 170. In a few colonies, one or several apparently younger, non-pigmented and non-physogastric neotenic females and males were found. Only the pigmented and physogastric neotenic females were selected for genotyping (table 1; electronic supplementary material, table S1). In a few rare cases, we directly observed the female nymphs of the fourth nymphal stage moulting into nymphoid neotenics with reduced wing pads, suggesting that the neotenic queens predominantly develop from the fourth nymphal stage.

Table 2. Newly developed polymorphic microsatellite markers. N_A, number of alleles; H_E, expected heterozygozity; H_O, observed heterozygozity; F_{IS}, inbreeding coefficient.

locus	motif	primer sequence (5′–3′)	label	N_A	allele size range (bp)	H_E	H_O	F_{IS}
PCR multiplex 1								
En-05[a]	$(tgg)_{12}$	F : ACGCAGCAGTAGGTACAGGAA	PET	5	145–166	0.343	0.140	0.601
		R : TCCAACCACAACCACTAGCA						
En-11	$(ac)_{14}$	F : CCAACTCGTAGGTGTAGAGGAT	NED	7	158–170	0.510	0.488	0.053
		R : CCGTCTCTTGTGAGTGTTGTG						
PCR multiplex 2								
En-08	$(ac)_{13}$	F : CTGAGCGGTTGCAGAGTACC	6′FAM	3	157–169	0.399	0.349	0.137
		R : TTCCCGGCCAAAGTACTAAC						
En-10	$(tg)_{14}$	F : CGTCCAGAAGATTCCTACCG	NED	7	103–135	0.697	0.744	−0.056
		R : TCTCTACCTCGTGTCTGCCT						
En-15	$(ca)_{15}$	F : CGATGAGATTCCGTAGACACC	NED	6	282–294	0.559	0.558	0.013
		R : AACCCTAGCACCTCACATGC						
En-19	$(tg)_{17}$	F : TACATTCAAATTAGTCTTGTGCCC	PET	6	177–195	0.579	0.605	−0.033
		R : TTGGTCGAGCCTATCTGGTC						
En-21[a]	$(tac)_{18}$	F : ACCATAACAACTACTACCACTACCACT	VIC	3	107–113	0.268	0.163	0.402
		R : TCGTAACTAAGTGTTGAATGTGAATTT						

[a]These loci were not used for the analysis of colony genetic structure.

(b) Microsatellite characteristics

Five of the seven tested loci had di-nucleotide repeat motifs and two had tri-nucleotide repeats (table 2). Loci showed three to seven alleles across the 43 tested colonies. None of the 21 pairs of loci showed significant gametic disequilibrium ($p > 0.119$) except for the pair En-05/En-21 ($p = 0.0130$). Observed heterozygosities ranged from 0.140 to 0.744 and expected heterozygosities from 0.268 to 0.697. Mean observed heterozygosity was 0.435 (s.d. = 0.228). Two loci, En-05 and En-21, showed significant heterozygote deficit when exact p-values were estimated by the Markov chain method ($p < 0.05$). None of the loci showed significant heterozygote excess or deficit after Bonferroni correction for multiple comparisons. Micro-Checker analyses indicated that the population was possibly in HWE, with loci En-05 and En-21 showing signs of a null allele (frequency = 0.23 and 0.14, respectively). We did not detect any evidence of scoring error because of large allelic dropouts. However, an excess of large homozygote classes at the locus En-05 indicated stuttering. Therefore, the loci En-05 and En-21 were excluded from the genetic analysis of colony structure and the analysis was carried out using the remaining five loci.

(c) Colony genetic structure

In soldiers and workers of all 10 colonies, we observed a maximum of four alleles and four different genotypes at each locus (electronic supplementary material, table S3). A single pair of reproductives in each colony was identified by the parental analysis for each locus (electronic supplementary material, table S3). The genotypes of sampled primary kings and queens always matched with those proposed by the reconstruction; thus, inferred and genotyped kings and queens referred to the same individuals. Since one or both primary reproductives were known and genotyped in all colonies, we were able to reconstruct the parental genotypes with high exclusion probabilities (p_{ex} for one parent known for all loci = 0.858; p_{ex} for parent pairs known for all loci = 0.966) and high probabilities of a correct assignment of paternal and maternal genotypes. For colonies with a known primary king and an inferred primary queen, the primary king had probabilities of reconstructed genotypes ranging from 0.993 to 1 and the inferred primary queen from 0.992 to 1. For the colony J with a known pair of primary reproductives, these values were equal to 1. The distribution of paternal and maternal alleles in the genotypes of the sterile castes was consistent with Mendelian distribution (G-test summed over loci: $p = 0.278$–0.990). The negative value of within-colony inbreeding ($F_{IC} = -0.326$, 95% CI, −0.437 to −0.218) was not significantly different from the value expected for simple families with a pair of monogamous reproductives [23]. The F_{IT} value (0.112, 95% CI, 0.056–0.177) indicated a significant level of inbreeding at the population level. The relatedness among the workers and soldiers in all colonies ($r = 0.575$) did not differ significantly from 0.5, a value expected for full siblings. All these observations suggest that the 10 studied colonies were founded as simple families by a pair of related primary reproductives and that the sterile castes were produced sexually.

In neotenic queens (219 genotyped individuals from nine colonies), we observed a maximum of two alleles and a maximum of three different genotypes per locus in each colony (electronic supplementary material, table S3). The neotenic queens always contained either both alleles of their mother in a heterozygous combination or one of the maternal alleles in a homozygous combination. By contrast, exclusively paternal alleles were never observed in the genotypes of neotenic queens. This suggests that the neotenic queens were produced asexually, through thelytokous parthenogenesis of their mothers.

Genotypes of nymphs indicated their mixed reproductive origin. Male nymphs showed a pattern similar to that of

Table 3. Transitions to homozygosity in parthenogenetic neotenic daughters for loci heterozygous in the inferred genotype of the founding primary queen. PQ_{HET}, number of inferred primary queens heterozygous at the locus; NF_{TOT}, number of neotenic parthenogenetic daughters from a heterozygous mother; NF_{HOMO}, number of homozygous neotenic daughters at the locus; *R*, observed rate of transition to homozygosity corresponding to the proportion of homozygous neotenic daughters) produced by heterozygous mothers; *r*, expected generational rate of transition to homozygosity; n.s., not significant.

						automixis			
locus	PQ_{HET}	NF_{TOT}	NF_{HOMO}	*R*	apomixis ($r = 0$)	gamete duplication ($r = 1$)	terminal fusion ($r = 0.33–1$)	central fusion ($r = 0–0.33$)	random fusion ($r = 0.33$)
En-11	7	180	3	0.02	***	***	***	n.s.	***
En-10	3	60	1	0.02	***	***	***	n.s.	***
En-08	4	119	10	0.08	***	***	***	n.s.	***
En-19	3	60	1	0.02	***	***	***	n.s.	***
En-15	3	60	3	0.05	***	***	***	n.s.	***

***χ^2-test, $p < 0.001$. When *r* is within a range of values, the value closest to the observed *R* was used for the test.

workers and soldiers, with a maximum of three alleles and four different genotypes at each locus in each colony, and a Mendelian distribution of alleles, indicating that they were sexually produced (electronic supplementary material, table S3). Genotypic patterns of female nymphs were more complex: while most of them were carrying parental alleles in a Mendelian distribution, six out of 18 females showed genotypes unexpected under sexual reproduction, with paternal alleles lacking at all loci. In other words, the samples of female nymphs contained individuals produced sexually as well as parthenogens of the queen(s) (electronic supplementary material, table S3).

(d) Restoration of ploidy

The relatednesses among the parthenogenetic neotenic queens ($r = 0.982$) and between the parthenogens and their inferred mothers ($r = 0.991$) were dramatically different from the value 0.5 expected under sexual origin of the neotenics. The parthenogenetic queens were not almost exclusively homozygous at all loci, as has been reported in the case of *Reticulitermes* [6–8]. Instead, we observed a high rate of conservation of heterozygosity in the parthenogens for the loci that were heterozygous in the inferred maternal genotypes, indicating that a different mode of thelytoky occurs in *E. neotenicus*. The rates of transition to homozygosity (*R*), listed in table 3, ranged from 2 to 8% for the five studied loci. These rates were consistent with the values expected under automixis with central fusion, a process yielding heterozygous genotypes when recombination is absent and homozygous genotypes only when recombination takes place.

4. Discussion

In this study, we demonstrate the occurrence of AQS in the higher termite *Embiratermes neotenicus* from the Neotropical subfamily Syntermitinae. This exceptional reproductive strategy, in which the queens combine sexual reproduction to produce the sterile castes and dispersing reproductives with asexual reproduction to produce non-dispersing neotenic queens, had previously been documented only in three species of a single genus of lower termites, the genus *Reticulitermes* [6–8]. We also have data suggesting that AQS occurs in another higher termite, *Cavitermes tuberosus* (Termitinae) [25].

The discovery of AQS in *Embiratermes* and *Cavitermes* adds to the list of breeding systems observed in the higher termites. The evolution of extreme queen physogastry and a lifelong monogamy of a single pair of primary reproductives are the dominant aspects of reproduction in most higher termites [10]. AQS may appear to be an antipode of the monogamy, with multiple queens participating in the reproduction of the colony. However, functionally, AQS represents an extension of the monogamous situation that goes even further in enhancing the reproductive potential of the founding primary queen; not only is the queen physogastric, but she also multiplies the transmission of her genes and the colony growth rate by the production of up to hundreds of highly related parthenogens, replacing her after her death in a virtually everlasting life cycle. At the same time, the production of parthenogens prevents inbreeding in subsequent generations of offspring that would arise from the mating of sexually produced neotenic queens with their own father or brothers, and thus conserves a high genetic diversity of helpers and dispersing reproductives. It is difficult to make direct inferences on how the AQS contributes to the ecological success of individual species. Nevertheless, it is noteworthy that *Embiratermes neotenicus* is often ranked among the most abundant Neotropical termite species in terms of colony sizes (up to over half a million individuals), local abundances and widespread distribution (throughout the entire Amazon basin and Guianas) [26–28].

In all 10 genotyped colonies, the gene pool appeared to come from a single pair of primary reproductives paired after the dispersal flight. All sterile individuals, workers and soldiers, were produced sexually by the mating of the primary king with the primary queen or her parthenogenetic daughters. Most of the fourth-stage male and female nymphs collected before the swarming season appeared to be sexually produced, while only six female nymphs were unambiguously described as parthenogenetic. Since the neotenic queens develop from the fourth nymphal stage, it is impossible to decide whether these six individuals were destined to become winged reproductives or neotenic queens.

Remarkably, the parthenogenetic queens were not produced through automixis with terminal fusion, yielding practically

perfect homozygotes at all loci, as documented in all three cases of AQS in *Reticulitermes* as well as in *Cavitermes tuberosus* [25]. On the contrary, the neotenic parthenogens showed a very low generational rate of transition to homozygosity (2–8%) for the loci heterozygous in their inferred mothers. Thus, thelytoky in *E. neotenicus* is based on a different cytological mechanism, not yet reported in termites. The observed frequencies of transition to homozygosity are consistent with automixis with central fusion, a mechanism of thelytoky reported in some social Hymenoptera (i.e. ants and Cape honeybees) [3,4]. In some of these hymenopterans, the central fusion is accompanied by a reduced rate of recombination and a practically complete conservation of heterozygosity. It is not the case in *E. neotenicus*, in which the rates of transition to homozygosity differ among loci and appear to reflect different probabilities of recombination events for each locus, depending on its position on the chromosome.

The reduction of heterozygosity in parthenogenetic queens of *E. neotenicus* is much lower than that in *Reticulitermes* parthenogens. Yet the gradual increase in homozygosity of neotenic queens across eventual multiple cycles of AQS within a single colony may have an impact on the fitness of the parthenogens. Nevertheless, it is believed that the costs related to homozygosity are relatively low in philopatrically reproducing queens that do not disperse and are continuously assisted by helpers when compared with winged dispersing forms undergoing independent colony foundation [10,24]. In addition, the systematic occurrence of parthenogenesis over long time scales would lead to a rapid purging of deleterious alleles through non-viable parthenogens on loci with a high probability of recombination, yielding homozygous genotypes [29].

Except for a single nest, very small in size and headed by the primary reproductives, all inspected colonies in which we succeeded in finding the reproductives contained multiple neotenic queens. This suggests that the replacement of the primary queen by its parthenogens is a non-accidental event and that it takes place early in the colony life cycle. The observations on the life cycle of *Reticulitermes speratus* indicate that between the occurrence of parthenogens and the death of the primary queen there is a transition stage during which the neotenics coexist with their mother and reproduce [7,10]. We did not observe such a transition stage in *E. neotenicus*, probably due to the lack of intermediary nests in our sampling. Paternal genotype reconstructions unambiguously indicated that the sampled primary kings were the fathers of sterile castes, suggesting that the rare non-pigmented neotenic males found in some nests did not intervene significantly in the reproduction of the colonies as long as the primary king was present. Therefore, given the theoretical lifespan reported for primary reproductives in some higher termites [30] and our observations of active primary kings in very large colonies, this stage of colony development may last for a long time and the takeover of reproduction by the neotenic kings may be rare.

While the presence of AQS in *E. neotenicus* was expected from the striking similarities between the breeding structures of this species and those of *Reticulitermes speratus*, the use of two different cytological mechanisms of parthenogenesis in different termite taxa is astonishing. A question therefore arises of whether the occurrence of AQS in individual lineages of lower and higher termites represents independent evolutionary events. Whatever is the correct answer, another intriguing question concerns the selection pressures favouring AQS given the dramatic ecological differences between xylophagous *Reticulitermes* termites from temperate regions on the one hand and tropical humivorous *Embiratermes* on the other hand. Vice versa, a number of closely related species with similar ecology unambiguously lack AQS, such as several *Reticulitermes* species [8,31] and *Labiotermes labralis*, a humivorous syntermitine sympatric with *E. neotenicus* [32]. At the same time, it must be noted that rigorous studies on the breeding systems of termites with large colony populations are scarce, especially in tropical humivorous species. Therefore, practically no data are available for other species of the genus *Embiratermes*, for instance. Moreover, the use of genetic markers to infer the breeding structure solely from the genotypes of workers or soldiers may be misleading and may indicate a simple family even in colonies with AQS.

In conclusion, along with the first descriptions of AQS in two unrelated genera of higher termites, *Embiratermes* and *Cavitermes*, a vast array of questions emerges that should be answered in the future, starting from an extensive survey of the occurrence of AQS and its cytological mechanisms across different subfamilies of Termitidae, to comparative analyses of ecological determinants correlated with the presence of AQS. Undoubtedly, new discoveries on the breeding systems of higher termites are to be expected, adding to the complexity of reproductive strategies of the oldest eusocial insects.

Data accessibility. The characteristics of the newly developed microsatellite markers, primer combinations used in the multiplexes and the genotypic frequencies observed in the studied colonies are included in table 2 and electronic supplementary material, table S3 of this article. COI haplotype sequences have been deposited in GenBank under accession numbers KP769532–35 and in Dryad Digital Repository under doi:10.5061/dryad.2t99d.

Authors' contributions. V.R., Y.R., D.S.-D., R.H. and C.P. designed the study, K.D., R.F., R.H., Y.R. and D.S.-D. collected the material, and R.F., V.R. and D.S.-D. analysed the genetic structure of colonies. All authors contributed significantly to the text of the manuscript and approved the final version.

Competing interests. We declare we have no competing interests.

Funding. This research was funded by the Belgian National Fund for Scientific Research (FRFC grant 2.4594.12), the Czech Science Foundation (14-12774S), the Grant Agency of the Charles University in Prague (580413) and the Institute of Organic Chemistry and Biochemistry, AS CR (RVO: 61388963).

Acknowledgements. We are grateful to P. Cerdan and the staff of the Laboratoire Environnement HYDRECO of Petit Saut (EDF-CNEH) for logistic support, to Prof. Evelyne Garnier-Zarli for academic support, and to Sophie Frechault and Marjorie Labédan for technical help. We thank two anonymous referees for their helpful suggestions.

References

1. Bell G. 1982 *The masterpiece of nature: the evolution and genetics of sexuality*. Berkeley, CA: University of California Press.
2. Schön I, Martens K, van Dijk P. 2009 *Lost sex: the evolutionary biology of parthenogenesis*. Dordrecht, The Netherlands: Springer.
3. Wenseleers T, Van Oystaeyen A. 2011 Unusual modes of reproduction in social insects: shedding light on the evolutionary paradox

of sex. *BioEssays* **33**, 927–937. (doi:10.1002/bies.201100096)

4. Rabeling C, Kronauer DJ. 2013 Thelytokous parthenogenesis in eusocial Hymenoptera. *Annu. Rev. Entomol.* **58**, 273–292. (doi:10.1146/annurev-ento-120811-153710)
5. Pearcy M, Aron S, Doums C, Keller L. 2004 Conditional use of sex and parthenogenesis for worker and queen production in ants. *Science* **306**, 1780–1783. (doi:10.1126/science.1105453)
6. Matsuura K, Vargo EL, Kawatsu K, Labadie PE, Nakano H, Yashiro T, Tsuji K. 2009 Queen succession through asexual reproduction in termites. *Science* **323**, 1687. (doi:10.1126/science.1169702)
7. Vargo EL, Labadie PE, Matsuura K. 2012 Asexual queen succession in the subterranean termite *Reticulitermes virginicus*. *Proc. R. Soc. B* **279**, 813–819. (doi:10.1098/rspb.2011.1030)
8. Luchetti A, Velonà A, Mueller M, Mantovani B. 2013 Breeding systems and reproductive strategies in Italian *Reticulitermes* colonies (Isoptera: Rhinotermitidae). *Insectes Soc.* **60**, 203–211. (doi:10.1007/s00040-013-0284-8)
9. Matsuura K. 2011 Sexual and asexual reproduction in termites. In *Biology of termites: a modern synthesis* (eds DE Bignell, Y Roisin, N Lo), pp. 255–277. Dordrecht, The Netherlands: Springer.
10. Myles TG. 1999 Review of secondary reproduction in termites (Insecta: Isoptera) with comments on its role in termite ecology and social evolution. *Sociobiology* **33**, 1–91.
11. Holmgren N. 1906 Studien über südamerikanische Termiten. *Zool. Jahrb.* **23**, 521–676.
12. Malausa T *et al*. 2011 High-throughput microsatellite isolation through 454 GS-FLX titanium pyrosequencing of enriched DNA libraries. *Mol. Ecol. Resour.* **11**, 638–644. (doi:10.1111/j.1755-0998.2011.02992.x)
13. Raymond M, Rousset F. 1995 GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249.
14. Rousset F. 2008 GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.* **8**, 103–106. (doi10.1111/j.1471-8286.2007.01931.x)
15. Weir BS, Cockerham CC. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370. (doi:10.2307/2408641)
16. Goudet J. 2001 *FSTAT, version 2.9.3, a program to estimate and test gene diversities and fixation indices*. Lausanne, Switzerland: Lausanne University.
17. Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P. 2004 MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538. (doi:10.1111/j.1471-8286.2004.00684.x)
18. Jones AG. 2005 GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. *Mol. Ecol. Notes* **5**, 708–711. (doi:10.1111/j.1471-8286.2005.01029.x)
19. Jones OR, Wang J. 2010 COLONY: a program for parentage and sibship inference from multilocus genotype data. *Mol. Ecol. Resour.* **10**, 551–555. (doi:10.1111/j.1755-0998.2009.02787.x)
20. Queller DC, Goodnight KF. 1989 Estimating relatedness using genetic markers. *Evolution* **43**, 258–275. (doi:10.2307/2409206)
21. Thorne BL, Traniello JFA, Adams ES, Bulmer M. 1999 Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. *Ethol. Ecol. Evol.* **11**, 149–169. (doi:10.1080/08927014.1999.9522833)
22. Konovalov DA, Manning C, Henshaw MT. 2004 KINGROUP: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. *Mol. Ecol. Notes* **4**, 779–782. (doi:10.1111/j.1471-8286.2004.00796.x)
23. Bulmer MS, Adams ES, Traniello JFA. 2001 Variation in colony structure in the subterranean termite *Reticulitermes flavipes*. *Behav. Ecol. Sociobiol.* **49**, 236–243. (doi:10.1007/s002650000304)
24. Pearcy M, Hardy O, Aron S. 2006 Thelytokous parthenogenesis and its consequences on inbreeding in an ant. *Heredity* **96**, 377–382. (doi:10.1038/sj.hdy.6800813)
25. Roisin Y, Hanus R, Fournier D. 2014 Asexual queen succession in soil-feeding termites (*Cavitermes tuberosus*). In *17th Congress of IUSSI, 13–18 July 2014, Cairns, Queensland, Australia*. See http://hdl.handle.net/2123/11069.
26. Vasconcellos A. 2010 Biomass and abundance of termites in three remnant areas of Atlantic Forest in northeastern Brazil. *Rev. Bras. Entomol.* **54**, 455–461. (doi:10.1590/S0085-56262010000300017)
27. Constantino R. 1992 Abundance and diversity of termites (Insecta: Isoptera) in two sites of primary rain-forest in Brazilian Amazonia. *Biotropica* **24**, 420–430. (doi:10.2307/2388613)
28. Constantino R. 1998 Catalog of the living termites of the New World. *Arq. Zool. S. Paulo* **35**, 135–231. (doi:10.11606/issn.2176-7793.v35i2p135-230)
29. Charlesworth D, Willis JH. 2009 The genetics of inbreeding depression. *Nat. Rev. Genet.* **10**, 783–796. (doi:10.1038/nrg2664)
30. Thorne BL, Breisch NL, Haverty MI. 2002 Longevity of kings and queens and first time of production of fertile progeny in dampwood termite (Isoptera; Termopsidae; *Zootermopsis*) colonies with different reproductive structures. *J. Anim. Ecol.* **71**, 1030–1041. (doi:10.1046/j.1365-2656.2002.00666.x)
31. Vargo EL, Husseneder C. 2011 Genetic structure of termite colonies and populations. In *Biology of termites: a modern synthesis* (eds DE Bignell, Y Roisin, N Lo), pp. 321–347. Dordrecht, The Netherlands: Springer.
32. Dupont L, Roy V, Bakkali A, Harry M. 2009 Genetic variability of the soil-feeding termite *Labiotermes labralis* (Termitidae, Nasutitermitinae) in the Amazonian primary forest and remnant patches. *Insect Conserv. Divers.* **2**, 53–61. (doi:10.1111/j.1752-4598.2008.00040.x)