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

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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

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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

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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

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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 MICRO-CHECKER 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.

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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

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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 non-physogastric 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 non-physogastric 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

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

Castes and stages present/nest characteristics	Queen maturity level	Social structure category: 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.

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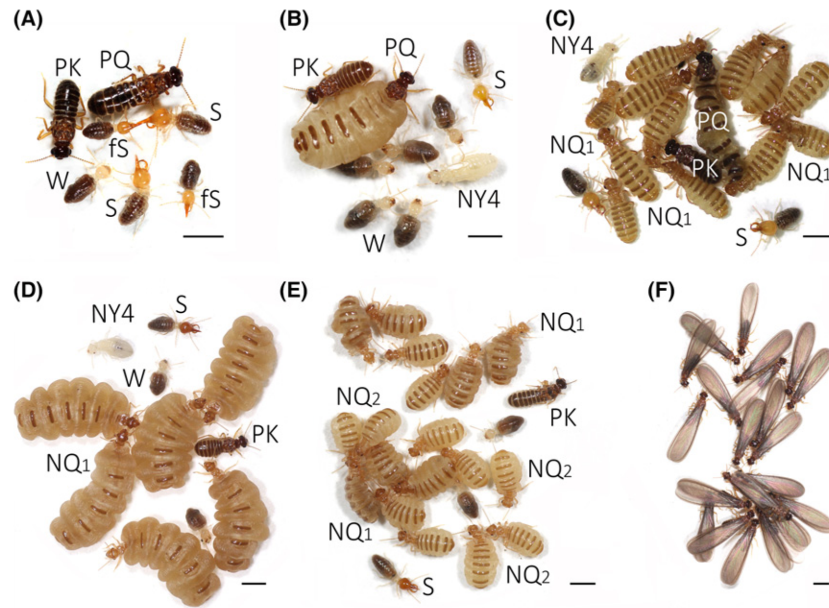

Fig. 1 Social structures of colonies in different stages of the life cycle based on observations of 137 colonies collected over three successive years in two different periods of the year. (A) Incipient primary colony ca. 4 months after colony foundation. (B) Advanced primary colony ca. 11 months after 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.

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

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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)

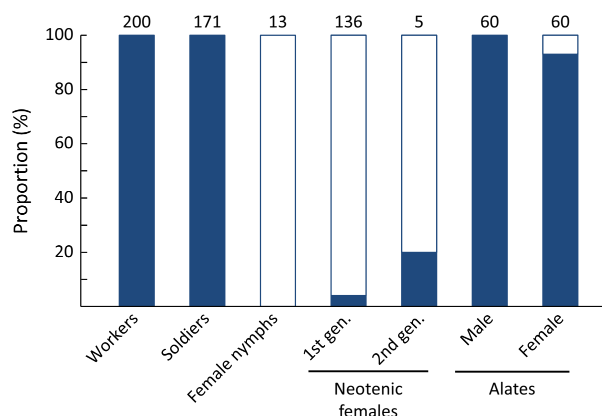

Fig. 2 Proportions of sexually (filled bars) and parthenogenetically (open bars) produced individuals in the 12 genotyped colonies, that is workers, soldiers, fourth-stage female nymphs from nondispersing colonies, two generations of neotenic females, and alate dispersers of both sexes. Numbers above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

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).

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

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

						Automixis			
Locus	PQ_{het}	NF_{tot} from PQ_{het}	NF_{hom}	*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)
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.
***$P < 0.001$.
**$P < 0.01$.

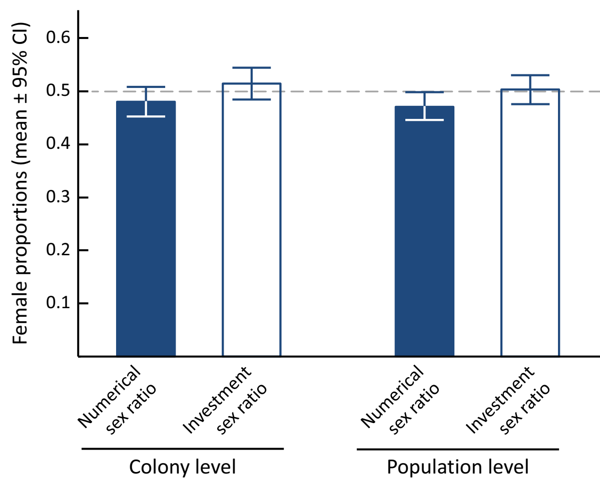

Fig. 3 Sex ratio of future alate dispersers calculated for 23 403 late stage nymphs and alates originating in 14 dispersing colonies, expressed as proportion of females. Filled bars show the numerical sex ratio, open bars show energetic investment into females, calculated as unweighted mean proportion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

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

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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

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(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.

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RESEARCH ARTICLE

Dispersal and mating strategies in two neotropical soil-feeding termites, *Embiratermes neotenicus* and *Silvestritermes minutus* (Termitidae, Syntermitinae)

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Abstract
Colony breeding systems and dispersal strategies of eusocial insects shape the genetic structure at the colony, but also at the population level. Most of the few molecular studies dedicated to termites suggest that winged reproductives disperse far enough to secure the formation of outbred founding pairs. However, these studies almost exclusively focused on wood-feeding termites and knowledge about the dispersal potential of winged reproductives is missing for soil-feeding termites. We investigated the dispersal and mating strategies of *Embiratermes neotenicus* and *Silvestritermes minutus* (Termitidae, Syntermitinae), two very abundant soil-feeding species from the Neotropics. In both species, analysis of microsatellite markers indicated low genetic similarity between closely located colonies and low genetic differentiation between populations separated by less than 10 km. Each of the 39 *E. neotenicus* colonies originated from a single pair of primary reproductives and the mean inbreeding coefficient of sterile castes was only slightly different from that expected in offspring of an outbred pair. Most *S. minutus* colonies (34/41) were consistent with outbred biparental foundation. In three mature colonies, the genotypes of sterile castes suggested their origin by mixing of multiple related reproductives. Finally, four colonies in late stage of the colony life cycle contained sterile populations originating from multiple unrelated reproductives. We conclude that long-distance flights resulting in outbred reproduction are common in these soil-feeding species in pristine habitats but that other factors, such as mating preferences, could increase relatedness between founders.

Keywords Termites · Population genetic structure · Dispersal · Breeding systems

Introduction

Genetic structures of colonies and populations of social insects are shaped by the breeding system as well as by dispersal strategies of individual species. In the majority of termite species, new colonies are founded by a pair of primary reproductives, which leave the natal nests as flying winged dispersers (alates). Colony foundation by accidental colony

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fragmentation or by budding of colony portions containing secondary reproductives is considered a rare alternative to foundation by alate dispersal flights (Vargo and Husseneder 2011). Such flights generally occur once to twice a year over a period of a few days to several weeks, and can be synchronized among conspecific nests over distances of several 100 km (Nutting 1969; Martius 2003). Synchronization is vital for the maintenance of outbreeding, together with sufficient dispersal distance and mechanisms of outbred mate preference (e.g. Shellman-Reeve 2001). Records on dispersal distance most often vary from a few to hundreds of meters, in some cases up to nearly 1 km (Messenger and Mullins 2005; Hu et al. 2007). Molecular studies on population genetic structure generally support the presumptions of dispersal by flying alates and suggest few budding events since genetic viscosity, i.e. colonies spatially close being genetically similar, was rarely demonstrated in natural populations (Vargo and Husseneder 2009; Vargo and Husseneder 2011). However, these studies are, with rare exceptions, dedicated to subterranean pest species of the Rhinotermitidae family.

Feeding on soil evolved from the ancestral wood-feeding strategy in the family Termitidae ("higher termites"). Soil-feeding species occur in several lineages of Termitidae and represent over one-third of all described termite species. Soil feeders are especially abundant and diversified in humid tropical forests, where they largely contribute to the soil humification process. Fundamental knowledge about the dispersal abilities of alates and (re)-colonization potential of habitats such as isolated patches or islands after perturbation is scarce in soil-feeding termites. It was proposed that soil feeders were poor passive dispersers over water gaps because their colonies are usually located in the soil or soil-made nests and are unlikely to raft over water gaps, contrary to wood feeders frequently drifting in wood pieces (Gathorne-Hardy et al. 2000; Eggleton and Tayasu 2001). Up to date, a single genetic study investigated the dispersal potential of alates in soil feeders in pristine habitats, i.e. Fournier et al. (2016) in *Cavitermes tuberosus*. Therefore, additional knowledge on dispersal and mating strategies is of interest in this ecologically important group.

In the present paper, we report on the colony and population genetic structure in two soil-feeding species with high local abundances and very large distribution areas throughout neotropical rainforests, namely *Embiratermes neotenicus* (Holmgren 1906) and *Silvestritermes minutus* (Emerson 1925) (Termitidae, Syntermitinae). Beside their typical soil-feeding ecology, our interest in the two species is further prompted by their unusual breeding system. We reported recently in both species a breeding strategy known as asexual queen succession (Fougeyrollas et al. 2015; Fougeyrollas et al. 2017). It was first described in *Reticulitermes speratus* (Rhinotermitidae) (Matsuura et al. 2009) and it is now established in several termite species (reviewed in Matsuura 2017). In this system, the founding primary queen is replaced by numerous neotenic daughters arising through thelytokous parthenogenesis. The neotenics then take over the reproduction of the colony and mate with the founder king. By contrast, all sterile colony members as well as most alate imagoes are produced by conventional sexual reproduction. The multiplication of female reproductives would allow an increase of the colony's reproductive potential and hence of its population and alate production, while avoiding inbreeding between reproductives.

While the breeding system of *E. neotenicus* and *S. minutus* is now well known, the reproduction of colonies, i.e. the dispersal and mating strategies, remain to be investigated. Our field observations and previous genetic analyses suggest that in spite of the large numbers of neotenics in colonies of both species, the dispersal of alate imagoes is the exclusive mode of colony foundation (Fougeyrollas et al. 2015; Fougeyrollas et al. 2017). High nest abundances and accessibility of their above ground parts in both species provide an opportunity to evaluate how mating and dispersal behaviors can shape the genetic structure both at small (< 10 km) and very small (< 300 m) spatial scales.

Materials and methods

Sampling and DNA extraction

Workers and soldiers from 40 *E. neotenicus* colonies were sampled in October 2012, May 2013 and March 2014 along an East–West axis (10 km) and a North–South axis (8 km), along the Route de Petit Saut and by the Sinnamary river in the vicinity of the Petit Saut Dam, French Guiana (N5°02.662′–5°07.202′, W53°03.295′–52°57.878′) (Fig. 1).

Workers and soldiers from 42 *S. minutus* colonies were sampled in April and November 2015, along the same East–West axis. Local abundance of *S. minutus* allowed a sampling scheme based on three sampling sites situated at similar latitudes (N5°04.3′–5°04.6′) and distant 3.7, 5, and 8.7 km one from each other, respectively (Fig. 2), with 14 colonies collected at each site. This sampling design was intended to provide an insight both into genetic structure at large spatial scale as well as into the patterns at very small scale (< 300 m).

Fifteen to 20 workers and/or soldiers per colony of *E. neotenicus* ($N = 795$) and 15 soldiers per colony of *S. minutus* ($N = 630$) were extracted using the DNeasy Blood & Tissue Kit (Qiagen, France) following the manufacturer's recommendations.

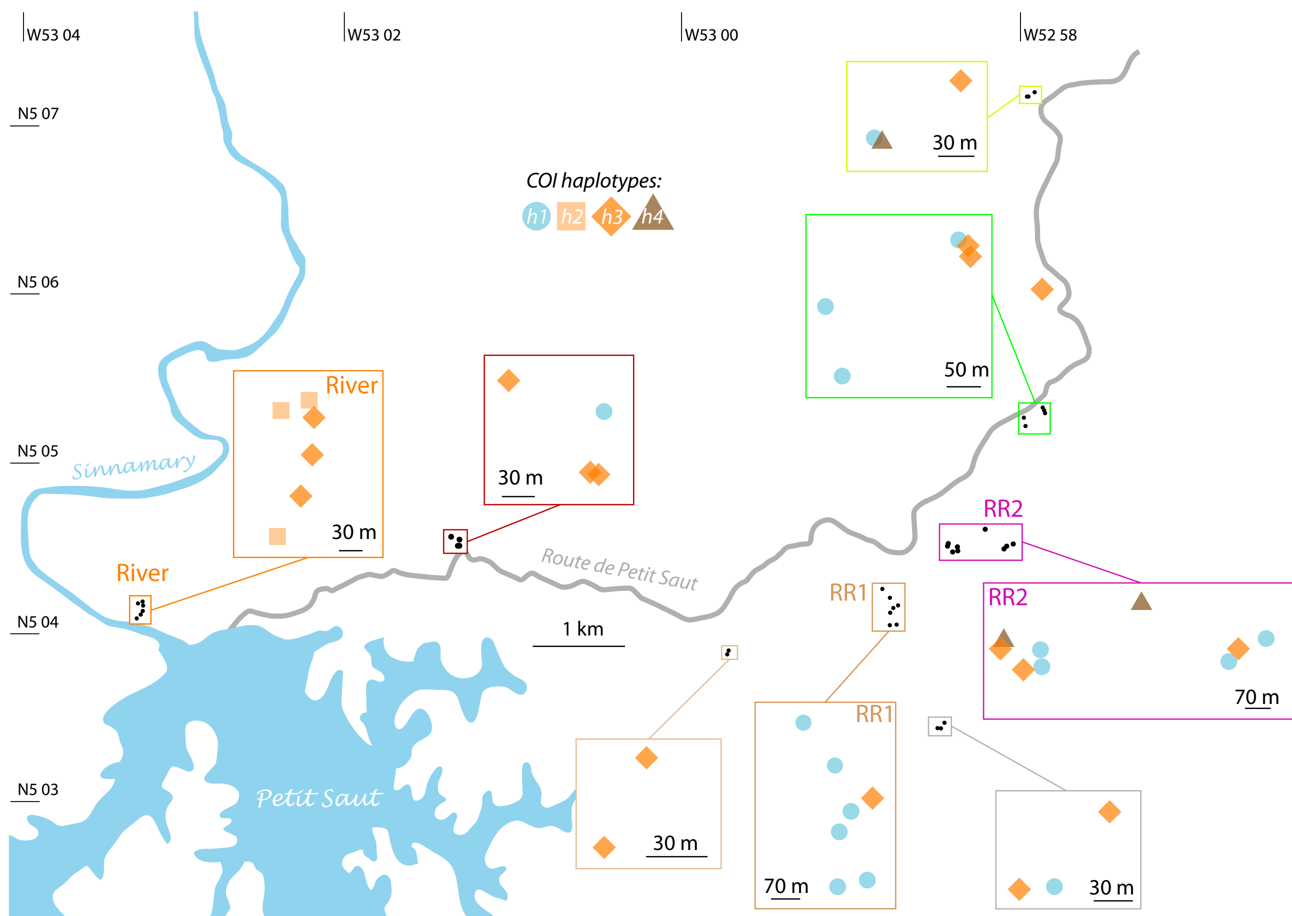

Fig. 1 Colonies of *Embiratermes neotenicus* and cytochrome oxidase I haplotype distribution reported on the map of Petit Saut, French Guiana. Colonies used for isolation by distance analysis were from the three sites indicated on the map: River, RR1 and RR2

Basic population statistics

For *E. neotenicus*, mitochondrial cytochrome oxidase I (standard barcoding region) sequences were previously obtained from one worker/soldier per colony (Fougeyrollas et al. 2015, GenBank Accession Numbers: KP769532–KP769535). Because pseudogenes were detected in cytochrome oxidase I sequences of *S. minutus*, mitochondrial cytochrome oxidase II was sequenced instead of the standard barcoding region following the protocol detailed in Roy et al. (2014), for one soldier per colony. These sequence data have been submitted to the GenBank database under AN MG725658–MG725663. *E. neotenicus* cytochrome oxidase I and *S. minutus* cytochrome oxidase II sequences were aligned using the MUSCLE algorithm implemented in Seaview 4.6. (Gouy et al. 2010). DnaSP 5.10.01 (Librado and Rozas 2009) and Arlequin 3.5.2 (Excoffier and Lischer 2010) were used to propose various estimators of the genetic diversity in a population: the number of haplotypes, haplotype diversity, number of variable sites, Nei's nucleotide diversity (1987) and Watterson's theta estimator (1975) for the total dataset for *E. neotenicus* and *S. minutus*, and for each sampling site for *S. minutus*.

E. neotenicus workers/soldiers were genotyped at 9 microsatellite loci: 5 loci were previously described (i.e. En08, En10, En11, En15 and En19) (Fougeyrollas et al. 2015) and 4 additional loci (i.e. En25, En35, En37 and En39) were developed for the present population genetic study (Supplementary Table S1). *S. minutus* soldiers were genotyped at 12 microsatellite loci previously described in Fougeyrollas et al. (2017). Microsatellite allele sizes were scored using GeneMapper 5.0 (Applied Biosystems, France).

In the first step, we excluded from further analyses one *E. neotenicus* sample and one *S. minutus* sample, both showing the genotypic pattern identical to that of the closest sampling point, suggesting repeated sampling of individuals originating from the same colony. Subsequently, different measures of genetic variation, i.e. number of alleles, Nei's unbiased expected heterozygosity, observed heterozygosity and fixation index, were calculated for each locus and over loci with Genetix 4.05.2 (Belkhir et al. 2004) and Fstat 2.9.3.2 (Goudet 2001), using a single worker/soldier from each colony

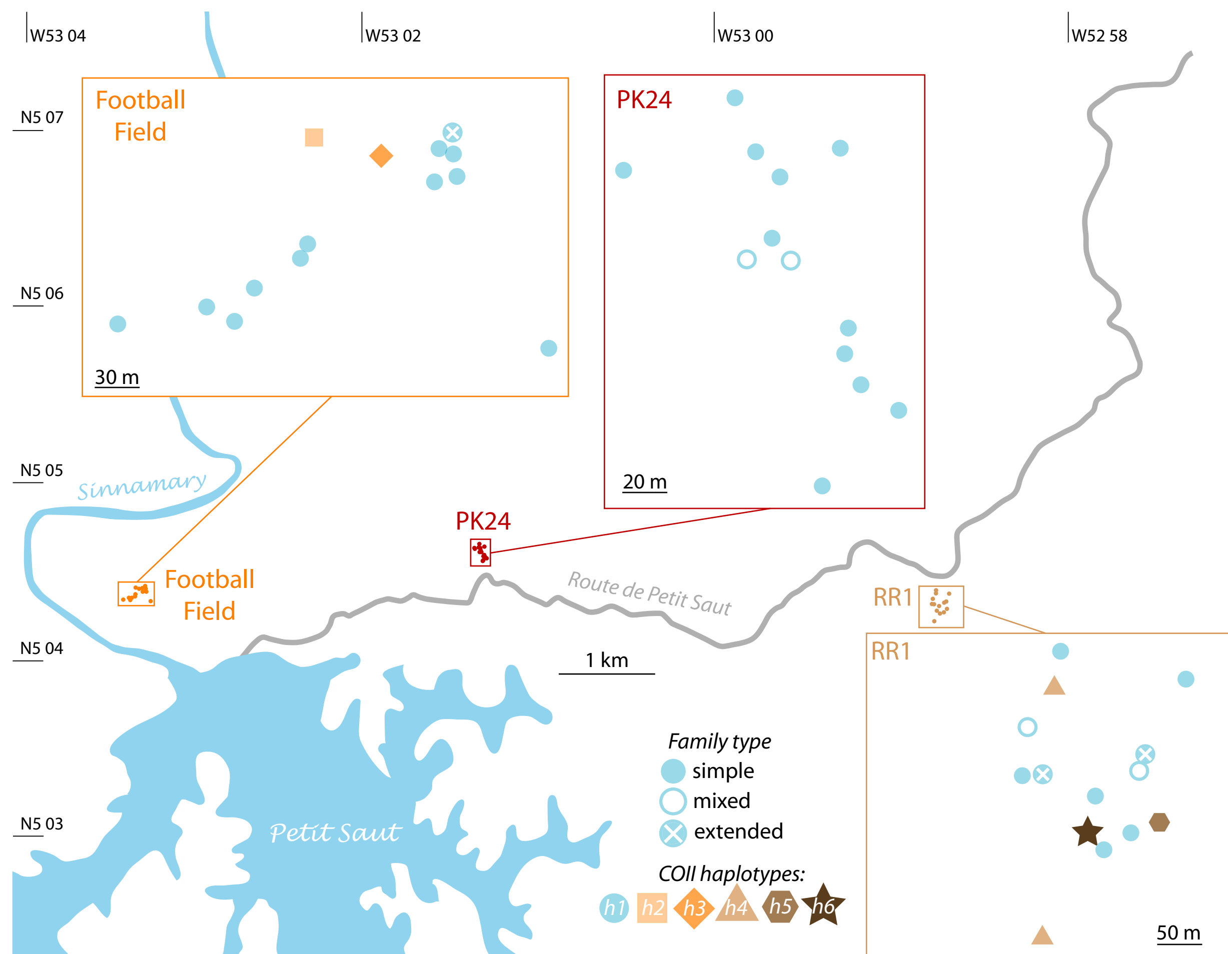

Fig. 2 Colonies of *Silvestritermes minutus* and cytochrome oxidase II haplotype distribution reported on the map of Petit Saut, French Guiana. Filled symbols represent simple families, open symbols mixed families, crossed symbols extended families. Colonies used for isolation by distance analysis were from the three sites indicated on the map: Football Field, PK24 and RR1

($N_{E.\ neotenicus}$ = 39 and $N_{S.\ minutus}$ = 41) in order to prevent within-colony relatedness bias. Exact tests for departures from the Hardy–Weinberg equilibrium for each locus and over loci, and linkage disequilibrium between all pairs of loci were performed using GENEPOP on the Web (Raymond and Rousset 1995). Genotyping errors due to null alleles, large allelic drop-outs or stutter bands were checked using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004).

Isolation by distance

Viscosity or genetic isolation by distance, the continuous increase in genetic differentiation among individuals with geographical distance due to limited dispersal, was assessed within three sites with the largest numbers of sampled colonies, i.e. River, RR1 and RR2 for *E. neotenicus* and Football Field, PK24 and RR1 for *S. minutus*. Ten replicated microsatellite datasets with a random single individual from each of the colonies were constructed and the significance of the correlations between genetic (Edwards' distances) and geographic distances (Euclidean distances) was tested using a Mantel test with 999 permutations using the package ADEGENET 2.0.1 in R 3.3.0 (Jombart 2008).

Bayesian clustering

Replicated microsatellite datasets were analyzed using two spatially explicit Bayesian clustering methods implemented in BAPS 6 (Corander et al. 2008) and in GENELAND package in R 3.3.0 (Guillot et al. 2005), in order to infer the number of genetic clusters and their spatial boundaries, and one non-explicit method implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). BAPS simulations were run ten times for each of $K = 1–10$ using population mixture analysis with the spatial clustering of individuals option. GENELAND simulations were run using the correlated frequencies model, K was allowed to vary from 1 to 10 and 100,000 MCMC iterations were run using a thinning interval of 100. STRUCTURE

simulations were run with the following settings: admixture model, allele frequencies correlated, a burn-in of 50,000 and 100,000 Markov chain Monte Carlo (MCMC) repetitions. Ten iterations were run for each assumed cluster ($K = 1$–10). Structure Harvester 0.6.94 (Earl and vonHoldt 2012) was used to determine the optimal K value following Delta K method (Evanno et al. 2005). Once the number of clusters was defined, all iterations were aligned using the Greedy algorithms implemented in Clumpp (Jakobsson and Rosenberg 2007).

Hierarchical F-statistics were estimated at different levels with the package hierfstat (Goudet 2005) in R 3.3.0: differentiation among colonies within the total population (F_{CT}), differentiation among colonies within highlighted Bayesian genetic clusters (F_{CG}) and differentiation between clusters within the total population (F_{GT}). Significance of F-statistics was assessed from 95% confidence intervals (CI) by bootstrapping over loci.

Colony genetic structure

We evaluated the maximum number of alleles and genotypes observed in each colony, and G-tests summed over loci were performed to compare observed genotype distribution to theoretical expectations under Mendelian distribution. We classified colonies into three family types according to Vargo et al. (2003) and DeHeer and Vargo (2004): "simple families" headed by a monogamous pair of reproductives (≤ 4 alleles and ≤ 4 genotypes at any locus, $P_{G\text{-test}} \geq 0.05$), "extended families" headed by multiple inbred neotenics produced within the nest (≤ 4 alleles and > 4 genotypes at one or more loci or $P_{G\text{-test}} < 0.05$) and "mixed families" headed or founded by multiple unrelated reproductives (> 4 alleles at one or more loci). In simple families, parental genotypes were reconstructed using a Punnett square. In order to further investigate biological origins of mixed families, sibships were investigated using ten additional genotyped soldiers per mixed family with Colony 2.0.6.3 (Jones and Wang 2010). Analyses were run without individuals carrying singleton alleles in order to exclude genotyping errors or sampling of solitary foreign individuals, so that reconstructed sib patterns correspond to a representative biological mixing of the colonies.

Inbreeding coefficients (F_{IC}, inbreeding within colonies; F_{IT}, inbreeding at the population level; F_{CT}, genetic differentiation among colonies) (Thorne et al. 1999) were calculated for the sterile castes and their significance tested by bootstrapping over loci with 1000 replications to generate 95% confidence intervals using Fstat. The relatedness among nestmates and, when possible, among reproductive pairs using reconstructed parental genotypes, was estimated using Relatedness 5.0.8. (Goodnight and Queller 1998). Estimates were bias corrected, and standard errors and 95% confidence intervals were calculated by jackknifing over loci and compared with the expected values.

Results

Basic population statistics

Cytochrome oxidase I dataset of *E. neotenicus* comprised 39 sequences of 653 bp, three polymorphic sites and four haplotypes (Table 1). The haplotype diversity was 0.646, the nucleotide diversity was 0.0012 and the Watterson's theta estimator was 0.0011. Haplotypes 1 and 3 were found both along the East–West and the North–South axes (Fig. 1). Haplotype 2 was only detected at the extreme West, near the Petit Saut Dam, while haplotype 4 was only found along the North–South axis, in the East of the study area.

Six haplotypes were identified for the full cytochrome oxidase II dataset of *S. minutus* (41 sequences), with five

Table 1 Basic population statistics estimated from microsatellite and mitochondrial datasets for the total population (*E. neotenicus* and *S. minutus*) and for each sampling site (*S. minutus*)

Sites	N_{ind}	Mitochondrial data					Microsatellite data				
		N_{hap}	$h \pm SD$	Nvs	pi ± SD	theta ± SD	N_a	H_E	H_O	p	F_{IS}
E. neotenicus											
All	39	4	0.646 ± 0.043	3	0.0012 ± 0.0001	0.0011 ± 0.0007	4.4	0.490	0.479	0.844	0.037
S. minutus											
All	41	6	0.273 ± 0.091	5	0.0004 ± 0.0001	0.0017 ± 0.0009	10.9	0.720	0.695	0.066	0.046
FF	14	3	0.275 ± 0.148	2	0.0004 ± 0.0002	0.0009 ± 0.0007	8.1	0.695	0.655	0.096	0.095
PK24	13	1	0	0	0	0	7.7	0.691	0.724	0.979	− 0.009
RR1	14	4	0.495 ± 0.151	3	0.0008 ± 0.0003	0.0014 ± 0.0009	7.7	0.689	0.708	0.465	0.009

N_{ind}, number of individuals; N_{hap}, number of mitochondrial haplotypes; *h*, haplotype diversity; *SD*, standard deviation; *Nvs*, number of variable sites; *pi*, Nei's nucleotide diversity; *theta*, Watterson's theta estimator; N_A, mean number of alleles per microsatellite locus; H_E, expected heterozygosity; H_O, observed heterozygosity; *p*, exact *p* value estimated by the Markov chain method for the Hardy–Weinberg test; F_{IS}, fixation index

variable sites on 678 bp (Table 1). The haplotype diversity was 0.273, the nucleotide diversity was 0.0004 and the Watterson's theta estimator was 0.0017. Haplotype 1 was recorded in all three sampling sites, haplotypes 2 and 3 were identified exclusively at Football Field, at the extreme West of the study area, and haplotypes 4, 5 and 6 were detected only at RR1, in the extreme East (Fig. 2).

All *E. neotenicus* microsatellite markers were polymorphic with a number of alleles per locus ranging from 2 to 7 (Supplementary Table S1). None of the nine loci deviated significantly from the Hardy–Weinberg equilibrium ($0.118 \leq p \leq 1$) and there was no significant gametic disequilibrium between the 36 pairs of loci ($0.168 \leq p \leq 1$), except for one pair (En11 and En25, $p = 0.011$). For the complete sample set, the mean values of expected and observed heterozygosity over loci were 0.490 and 0.479, respectively, and no deviation from the Hardy–Weinberg equilibrium was detected ($p = 0.844$) (Table 1).

The number of alleles per locus in *S. minutus* varied from 2 to 18 (Supplementary Table S2). No significant deviation from the Hardy–Weinberg equilibrium was observed for any of the loci ($0.053 \leq p \leq 0.781$) and none of the 66 pairs of loci was in significant linkage disequilibrium ($0.187 \leq p \leq 1$), except for one pair (Lal5 and Sm27, $p = 0.045$). The mean values for expected and observed heterozygosity over loci were 0.720 and 0.695, respectively, for the full sample set and no deviation from the Hardy–Weinberg equilibrium was observed ($p = 0.066$) (Table 1). When considering each sampling site separately, the mean number of alleles per locus was 8.1 for Football Field, 7.7 for PK24 and 7.7 for RR1. The mean values for expected/observed heterozygosity for all loci were 0.695/0.655, 0.691/0.724 and 0.689/0.708 for Football Field, PK24 and RR1, respectively, and no deviation from the Hardy–Weinberg equilibrium was detected ($0.096 \leq p \leq 0.979$) (Table 1).

Isolation by distance

No significant correlation was found between genetic differentiation and geographical distance among *E. neotenicus* colonies of Petit Saut for 9/10 replicated datasets of the River site (Mantel tests: $r = -0.445$ to 0.235, $p > 0.05$, 999 permutations), 10/10 replicated datasets of RR1 ($r = -0.028$ to 0.373, $p > 0.05$, 999 permutations) and 9/10 replicated datasets of RR2 ($r = -0.124$ to 0.250, $p > 0.05$, 999 permutations) (Fig. 3). Similarly, no significant evidence for isolation by distance was detected among *S. minutus* colonies for 9/10 replicated datasets of the Football Field site (Mantel tests: $r = -0.057$ to 0.196, $p > 0.05$, 999 permutations), for 10/10 replicated datasets of PK24 ($r = -0.131$ to 0.180, $p > 0.05$, 999 permutations) and 10/10 replicated datasets of RR1 ($r = -0.116$ to 0.196, $p > 0.05$, 999 permutations) (Fig. 3).

Population genetic structure

For both *E. neotenicus* and *S. minutus* microsatellite replicated datasets, Structure showed no evidence for population structure (data not shown). The optimal value of K, determined by the Delta K method, was different depending on the replicates. Whatever the K retained, the posterior probabilities of assignment of each individual were distributed across all of the K clusters.

Bayesian spatial analyses with Baps returned the probabilities of one cluster comprised between 0.857 and 1.000 for 10/10 *E. neotenicus* replicated microsatellite datasets, and between 0.684 and 0.999 for 8/10 *S. minutus* replicates. The analyses of the two remaining *S. minutus* replicates identified three clusters, independent of the sampling sites, with probabilities of 0.764 and 0.506. In these cases, each of the two additional clusters was constituted by only one colony.

Geneland analyses identified two genetic clusters ($K = 2$) for 9/10 *E. neotenicus* microsatellite replicated datasets and three clusters ($K = 3$) for 1/10 replicated dataset (Table 2). For $K = 2$, colonies from the extreme West were assigned to the first cluster and the remainder of the colonies were assigned to the second cluster (Fig. 4). For the majority of *S. minutus* replicated datasets, i.e. 7/10, Geneland identified two genetic clusters ($K = 2$), and three clusters ($K = 3$) for the remaining three replicated datasets (Table 2). For $K = 2$, colonies from the extreme West of the study area (Football Field site) were assigned to the first cluster and the remainder of the colonies (PK24 + RR1 sites) were assigned to the second cluster for 6/10 replicated datasets (Fig. 4).

The genetic differentiation was estimated using the two Bayesian clusters retrieved by Geneland for the majority of replicates in both species, i.e. the small Western cluster and the large Eastern cluster. For *E. neotenicus*, hierarchical F-statistics returned significant values for F_{CT}=0.3123 (95% CI 0.2756–0.3430), for F_{CG}=0.2597 (95% CI 0.2468–0.2714) and for F_{GT}=0.0711 (95% CI 0.0249–0.1134). Following the classification of Wright (1978), this last value corresponds to moderate genetic differentiation between *E. neotenicus* colonies of the small Western cluster and the large Eastern cluster.

For *S. minutus*, hierarchical F-statistics analyses indicated significant values for F_{CT} (0.2718, 95% CI 0.2592–0.2834), for F_{CG} (0.2549, 95% CI 0.2438–0.2668) and for F_{GT} (0.0227, 95% CI 0.0103–0.0360). The global F_{GT} value suggested a low genetic differentiation between the two clusters.

Genetic structure of colonies

For each of the 39 *E. neotenicus* colonies, a maximum of four alleles and four genotypes at each locus was observed in soldiers/workers. Mendelian distribution of parental alleles

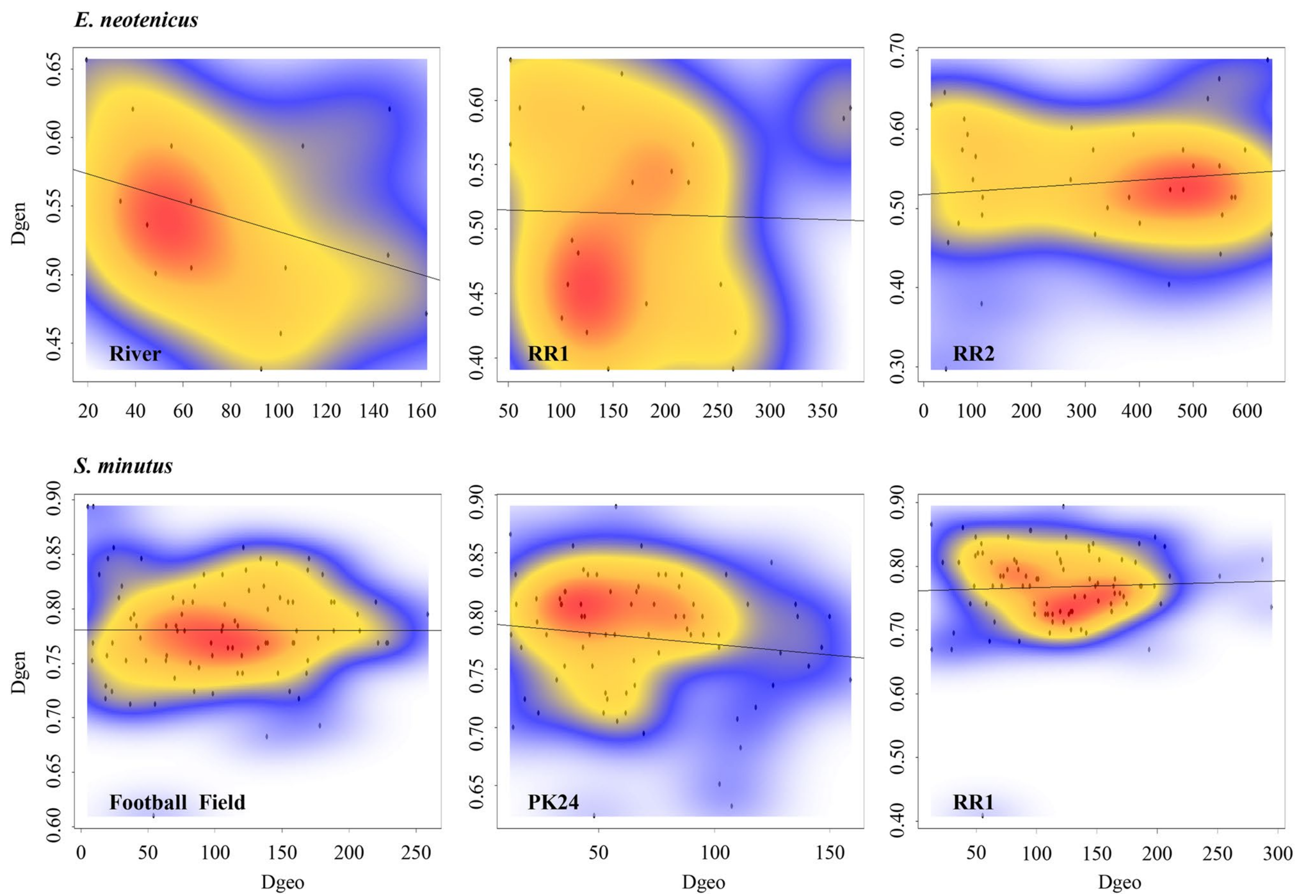

Fig. 3 Example of plots obtained for the isolation by distance analysis. Local density of points was plotted using a two-dimensional kernel density estimation. *Dgeo* geographical distances in meters, *Dgen* genetic distances. Analyses were realized for the sites of River, RR1 and RR2 for *E. neotenicus* and for the sites of Football Field, PK24 and RR1 for *S. minutus*

Table 2 Results from the spatially explicit Bayesian clustering method implemented in GENELAND for the ten microsatellite datasets of *E. neotenicus* and *S. minutus*

	E. neotenicus	*S. minutus*
Replicate 1	$K=2$ (*w*/*E*)	$K=3$ (FF/PK24/RR1)
Replicate 2	$K=2$ (*w*/*E*)	$K=2$ + "1" (*w*/*E*=FF/PK24+RR1)
Replicate 3	$K=2$ (*w*/*E*)	$K=3$ (FF/PK24+RR1/RR1)
Replicate 4	$K=2$ (*w*/*E*)	$K=2$ (*w*/*E*=FF/PK24+RR1)
Replicate 5	$K=3$ (*w*/*c*/*E*)	$K=3$ (FF/PK24/RR1)
Replicate 6	$K=2$ (*w*/*E*)	$K=2$ (*w*/*E*=FF/PK24+RR1)
Replicate 7	$K=2$ (*w*/*E*)	$K=2$ (*w*/*E*=FF/PK24+RR1)
Replicate 8	$K=2$ (*w*/*E*)	$K=2$ (FF+RR1/PK24)
Replicate 9	$K=2$ (*w*/*E*)	$K=2$ + "1" (*w*/*E*=FF/PK24+RR1)
Replicate 10	$K=2$ (*w*/*E*)	$K=2$ + "1" (*w*/*E*=FF/PK24+RR1)

K, number of inferred clusters; + *"1"*, "ghost" cluster without individuals assigned within; *w*, small western cluster; *E*, large eastern cluster; *c*, central cluster

within colonies (*G*-tests across loci, $p=0.081$–0.995) and strongly negative F_{IC} value (− 0.328, 95% CI − 0.358 to − 0.297) were consistent with a "simple-family" organization (Table 3). The high F_{CT} value (0.275, 95% CI 0.257–0.288) indicated genetic differentiation between colonies. The inbreeding coefficient of simple families (F_{IT}) was equal to 0.037 (95% CI 0.019–0.055) indicating low but significant inbreeding within colonies. Mean relatedness among sterile nestmates (*r*) was slightly superior to 0.5 ($r=0.529$, 95% CI 0.495–0.563). Mean relatedness within the inferred reproductive pairs was equal to 0.039 (95% CI − 0.091 to 0.1693).

For 34/41 *S. minutus* colonies and all microsatellite loci, a maximum of four alleles and four genotypes were recorded in sterile castes. Observed frequencies of neutral genotypes did not deviate from expected values under Mendelian distribution of paternal and maternal alleles (*G*-tests across loci, $p=0.236$–1). The F_{IC} value was strongly negative (− 0.356, 95% CI − 0.375 to − 0.335), suggesting a low number of reproductives, and the F_{IT} value was not significant (0.004, 95% CI − 0.017 to 0.027), indicating the

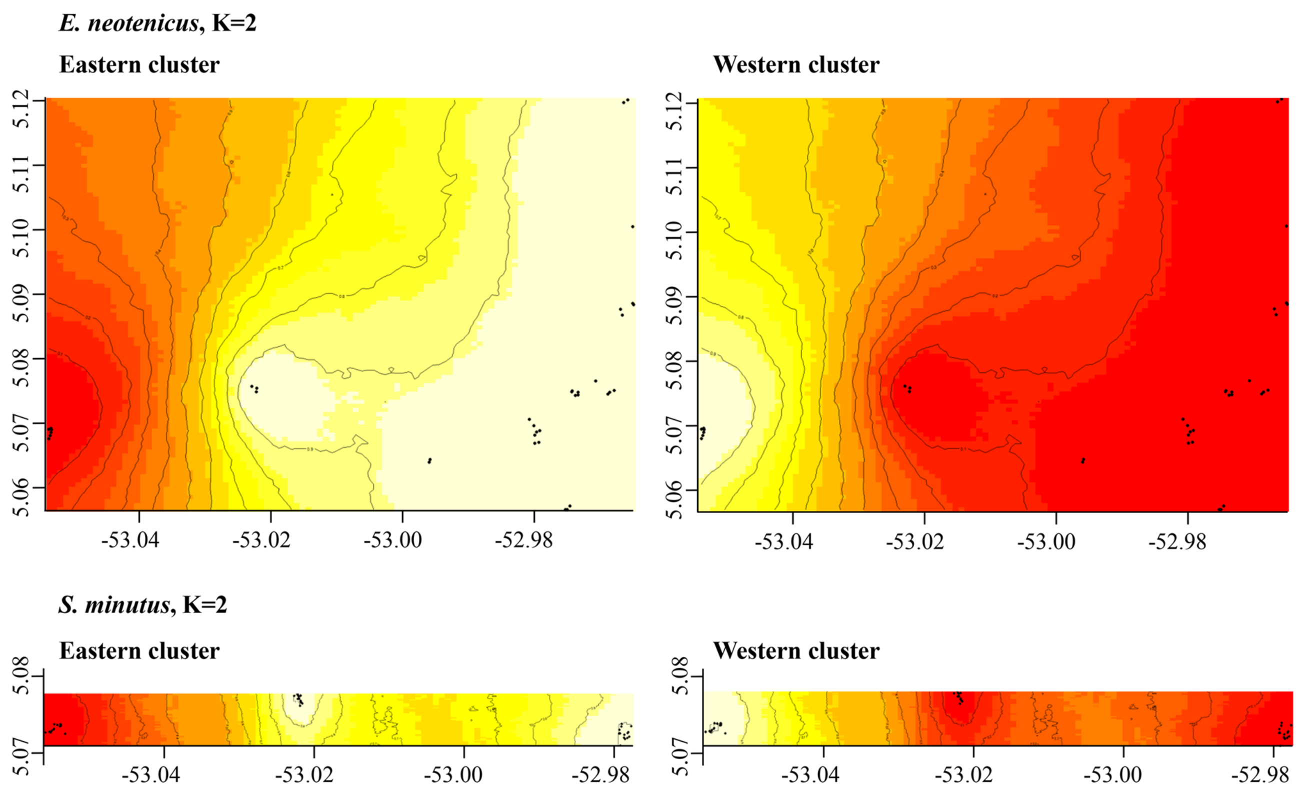

Fig. 4 Population structure of *E. neotenicus* and *S. minutus* colonies as estimated by the GENELAND analyses. Maps of posterior probabilities of the Western and Eastern clusters ($K=2$) for *E. neotenicus* and *S. minutus*. The highest membership values are in light tone and the level curves illustrate the spatial changes in assignment values

Table 3 *F*-statistics and relatedness coefficient in *E. neotenicus* and *S. minutus* for simple families, extended families and mixed families

	F_{IT}	F_{CT}	F_{IC}	r
E. neotenicus				
Simple families $N=39$	0.037 (0.019–0.055)	0.275 (0.257–0.288)	– 0.328 (– 0.358 to – 0.297)	0.529 (0.495–0.563)
S. minutus				
Simple families $N=34$	0.004 (– 0.017–0.027)	0.266 (0.252–0.281)	– 0.356 (– 0.375 to – 0.335)	0.519 (0.493–0.544)
Extended families $N=3$	0.158 (0.072–0.244)	0.354 (0.294–0.416)	– 0.303 (– 0.347 to – 0.252)	0.602 (0.510–0.684)
Mixed families $N=4$	– 0.001 (– 0.034–0.039)	0.174 (0.149–0.200)	– 0.212 (– 0.237 to – 0.181)	0.308 (0.270–0.345)

95% confidence intervals are indicated in brackets

N, number of colonies; F_{IT}, inbreeding at the population level; F_{CT}, genetic differentiation among colonies; F_{IC}, inbreeding within colonies; *r*, relatedness coefficient

absence of inbreeding (Table 3). Relatedness among sterile nestmates was $r=0.519$ (95% CI 0.493–0.544) and relatedness between individuals of inferred reproductive pairs was equal to – 0.044 (95% CI – 0.079 to – 0.009). These results suggested a simple family organization with an outbred pair of founding reproductives. For 3/41 colonies, we observed a maximum of four alleles but more than four genotypes for at least two loci, a significant F_{IT} value (0.158, 95% CI 0.072–0.244), a strongly negative F_{IC} value (– 0.303, 95% CI – 0.347 to – 0.252) and a high relatedness among sterile nestmates ($r=0.602$, 95% CI 0.510–0.684) (Table 3). This pattern corresponds to extended families. In 4/41 colonies, more than four alleles were recorded for at least three microsatellite loci and more than four genotypes were observed

for at least 5/12 loci, a pattern consistent with mixed families and confirmed by *F*-statistics (Table 3). Therefore, ten additional soldiers were genotyped in these colonies to infer sibships and parental genotypes based on a larger number of offspring. In two colonies, two sibships could be reconstructed, produced by three reproductives (one reproductive shared by both sibships and one opposite sex-reproductive specific to each sibship). In one colony, four sibships were reconstructed, three sharing a reproductive and one produced by a couple of independent parents. In the last colony, two sibships were reconstructed, each produced by one independent couple of parents. In mixed families, relatedness between sterile nestmates was equal to 0.308 (95% CI 0.270–0.345), relatedness between inferred opposite sex-reproductives was equal to − 0.120 (95% CI − 0.215 to − 0.024), and relatedness between same-sex reproductives was equal to 0.021 (95% CI − 0.0785 to 0.1213).

Discussion

Population genetic structure can arise at fine spatial scales when species have limited dispersal, leading to close spatial associations between relatives. In subterranean termites, short dispersal flights and/or budding have seldom been supported by genetic data (e.g. Baudouin et al. 2017; Perdereau et al. 2013, see Vargo and Husseneder 2011 for a review). The absence of isolation by distance was recently reported in the humivorous *Cavitermes tuberosus*, suggesting a relatively long-range dispersal (Fournier et al. 2016). Likewise, we did not observe fine-scale population viscosity in *Embiratermes neotenicus* and *Silvestritermes minutus*. Dispersal mediated by human transportation or rafting is very unlikely in soil feeders because reproductives are never found in human-manufactured or infested pieces of wood, unlike xylophagous termites (García et al. 2002; Brandl et al. 2005). Bourguignon et al. (2009) suggested that high elevation flight was an important feature for long dispersal distance in tropical rainforest termites. Termitidae alates were abundant in the canopy of a Panamanian forest and were caught up to a height of 28 m. By flying high, alates may take advantage of air currents above the upper canopy, and passive drift by winds could represent an important component of dispersal.

In the absence of barriers to dispersal, high rates of gene flow between sampled sites were thus expected in *E. neotenicus* and *S. minutus*. At a larger scale, i.e. ~ 10 km, clustering analyses indicated no or very weak spatial variation in genetic constitution for both species. Analyses conducted with Structure and Baps programs failed in detecting population genetic structure, including when Structure was allowed to make use of information about sampling locations. On the contrary, Geneland program was able to detect two spatial clusters for the majority of the replicated datasets, i.e. a small Western cluster composed of colonies established in the vicinity of the Sinnamary River and the dam, and a large Eastern cluster composed of all remaining colonies. Genetic differentiation between these clusters was low but significant. It has been previously postulated that when the data contain relatively little information, non-spatial Bayesian clustering algorithms sometimes do not provide a clear indication of population structure even when datasets show significant F_{ST} values between samples of individuals collected at different locations (Hubisz et al. 2009). Based on simulation studies, the Geneland model seems appropriate to infer recent linear geographical boundaries to gene flow, such as consequences of recent fragmentation and habitat destruction (Blair et al. 2012). Landscape features and habitat connectivity are known to influence dispersal patterns and genetic structure among natural populations. The relative genetic isolation of the Western *S. minutus* colonies agrees with the geographical isolation of the Football Field site, situated in a deep and flat, regularly inundated valley, surrounded by slopes of up to 30 m of height. Furthermore, two close physical barriers at the extreme West of the road, the river and the dam, and the associated activity, i.e. flooding, roads connecting the dam and exterior lightings around the dam area, could have acted or currently act as potential obstacles to gene flow between Western colonies and the remainder of Petit Saut colonies.

Highly variable loci such as microsatellites are efficient for studies of fine-scale genetic structure and represent the most suitable markers for investigations regarding the consequences of recent or contemporary (ongoing to a few tens of generations) landscape-level habitat modifications (Wang 2010; Wang 2011). On the opposite, maternally inherited mitochondrial markers, which evolve at slower rates, generally lack the resolution to reveal fine scale-structure and are thus best suited for inferring historical processes over hundreds to thousands of generations and large spatial scales. Therefore, the combined use of markers with different modes of inheritance can reveal differences in the long-term accumulated effects of sex-bias in dispersal (Anderson et al. 2010). Interestingly, the slight genetic differentiation obtained with microsatellite data for the Western colonies seems corroborated by the geographic distribution of mitochondrial haplotypes. The presence of unique haplotypes both at the extreme West and East of the Petit Saut Road (*E. neotenicus*: haplotypes h2 vs. h4 and *S. minutus*: haplotypes h2 and h3 vs. h4, h5 and h6) suggests that the spatial patterns observed could be more ancient than the construction of the dam (1989–1994). Additional genetic investigations deserve to be conducted at much larger geographical scales, in order to disentangle the direct effects of landscape structure from historical barriers to gene flow.

Our data on colony genetic structure also provided clues to understand mating patterns between primary reproductives, in connection with dispersal abilities. In 100% of *E. neotenicus* colonies and 83% of *S. minutus* colonies, the genotypes of sterile castes were consistent with expectations for simple families, indicating that colonies were headed by a single pair of primary reproductives. At the same time, the genetic constitution was consistent with mating of the founding primary king with a harem of neotenic queens, parthenogenetically produced by the foundress. Such a breeding system, called asexual queen succession, was recently documented in the two studied species (Fougeyrollas et al. 2015; Fougeyrollas et al. 2017). Molecular studies in various species of termites reported that relatedness between reproductive individuals of simple families may vary among species, and among populations in a species (reviewed in Vargo and Husseneder 2011). Accordingly, the inbreeding pattern was different in the two species. No significant inbreeding was detected in *S. minutus* steriles and relatedness values between inferred parents of simple families was close to zero, confirming outbred origin of primary reproductives following long-range dispersal. By contrast, the F_{IT} value for *E. neotenicus* steriles in simple families was significantly different from that expected for the offspring of a totally outbred pair of reproductives, indicating that some colonies could be headed by a pair of slightly related reproductives.

Inbreeding in simple families is generally explained by the pairing of related primary reproductives following short-range and/or asynchronous flights between colonies. However, as discussed above, no evidence was found for this hypothesis in *E. neotenicus*. Similar results were found for *Coptotermes lacteus*, a Rhinotermitidae building large mounds in Australia, in which primary inbreeding was not associated with viscosity (Thompson et al. 2007). Another hypothesis to explain deviations from the Hardy–Weinberg equilibrium in *C. lacteus* was a preference for non-sibling relatives over totally unrelated mates, i.e. 'optimal outbreeding' (Bateson 1983). Indeed, mating with genetically too distant partners can reduce the fitness of the offspring and lead to an outbreeding depression (Charlesworth and Willis 2009), while reasonably inbred matings could have positive effects on the inclusive fitness of the parents by increasing their representation of genes identical by descent in future generations. Thus, individuals should either avoid or favor inbred matings in order to balance the cost of inbreeding depression and the benefit of inbreeding (Kokko et al. 2006). A last hypothesis to explain inbred simple families is the presence of couples of related reproductives, implying a primary and a neotenic, or two neotenics produced by sexual reproduction (Vargo and Husseneder 2011). This hypothesis is particularly supported when reproductives are not collected and when genotypes of the reproductive pair are inferred from genotypes of steriles. In the case of *E. neotenicus*, genetic data were previously obtained for reproductive individuals only for a restricted number of colonies, but the contribution of sexually-produced neotenic individuals to offspring was never demonstrated (Fougeyrollas et al. 2015).

A small proportion of *S. minutus* colonies (3/41) was evaluated as extended families. In the formal definition, extended families are colonies headed by more than two reproductives and arise from inbreeding between parents and offspring or from brother–sister inbreeding. Our direct observations on the breeding structure indicated that all these three cases were colonies containing one primary king and a harem of neotenic females. We showed previously that a small proportion of sexually produced neotenic queens occurs in some colonies (5% of all genotyped neotenic females) (Fougeyrollas et al. 2017). Therefore, the observed inbreeding in the three colonies may likely be due to incestuous mating between the primary king and his daughters, i.e. neotenic females of sexual origin.

Finally, the genotypes in 4/41 *S. minutus* colonies indicated a mixed family structure, with the gene pool originating in at least three unrelated colony founders. Interestingly, three of these colonies were dispersing colonies, containing hundreds of alate dispersers together with a population of sterile castes, but no functional reproductives and young brood or eggs. The fourth colony was a large post-dispersal colony without alates, functional reproductives and young brood. The social composition of these colonies was in line with our proposed life cycle dynamics in *S. minutus*, characterized by a single massive dispersal of alates 3 years after colony foundation, preceded by the disappearance of reproductives and followed by a gradual decline of the sterile population in most colonies (Fougeyrollas et al. 2017). Our present observations suggest mixing of the original colony population with neighboring colonies during the pre-dispersal and/or post-dispersal period, following the disappearance of the reproductives. In particular, one of the dispersing colonies showed a very clear genetic pattern corresponding to the mixing of two colonies, with two full-sib sets, each produced by a couple of parents. It is difficult to judge how frequent are the mixings of unrelated colonies in declining nests and whether they represent veritable colony fusions or only exploitation of nesting and foraging resources by neighboring prospering colonies. Nevertheless, lowered agonism resulting in colony fusions in specific phases of the life cycle and particular seasons were previously reported to occur in some species (Clément 1986; Kaib and Brandl 1992; Bulmer and Traniello 2002), including acceptance of non-nestmates by colonies with a high proportion of nymphs (Matsuura and Nishida 2001). The other three dispersing colonies showed a more complex genetic pattern that can be explained by the contribution of at least two same-sex reproductives and a shared opposite-sex reproductive. Theory predicts that

mixed families can also arise when alates initiate colonies in cooperative groups of unrelated individuals (pleometrosis) (Thorne 1984; Darlington 1985; Hacker et al. 2005) or when they infiltrate mature, unrelated colonies immediately after dispersal. Adoption of foreign reproductives was reported experimentally in colonies where nymphs were absent (Neoh et al. 2012). Although our field observations do not suggest the adoption of reproductives, we could not totally rule out this last hypothesis. Indeed, our *F*-statistics were in line with models proposed by Thorne et al. (1999) and Bulmer et al. (2001) for adoption of unrelated reproductives, i.e. low F_{CT}, negative F_{IT}, negative F_{IC} and $r<0.5$.

Despite the abundance and species richness of soil-feeding termites in tropical forests and their positive impact on the organic matter cycling, we are far from understanding the dynamics of their dispersal and colonization capacities and their mate choice mechanisms. Recent research indicates that the breeding systems, another factor shaping the population genetics, may also be particularly diversified in the tropical soil feeders, including the previously unexpected cases of asexual queen succession strategies. The present study focuses on fine-scale population genetics of two sympatric species using these strategies and known for their large distribution areas in Neotropics. Comparative studies on population genetics of related (e.g. congeneric) sympatric species with a traditional breeding structure might be of particular interest to decipher the impact of the unusual breeding systems on the genetics of populations.

Data availability

We have deposited the primary data underlying these analyses as follows:

- DNA sequences: GenBank accessions MG725658–MG725663.
- Microsatellite characteristics uploaded as online Supplementary Material

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Sex-Pairing Pheromones in Three Sympatric Neotropical Termite Species (Termitidae: Syntermitinae)

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Abstract

Termite colonies are almost always founded by a pair of winged dispersers, in spite of the high costs and low success rates inherent in independent colony foundation. The dispersal flights of imagoes from natal colonies are followed by mate search, mediated by sex-pairing pheromones. Here, we studied the chemistry of sex-pairing pheromones and the related aspects of mate search in winged imagoes of two facultatively parthenogenetic species, *Embiratermes neotenicus* and *Silvestritermes minutus*, and an additional species from the same subfamily, *Silvestritermes heyeri*. All three species are widespread in the Neotropics, including the rainforests of French Guiana. After the dispersal flight and spontaneous loss of wings, females expose their hypertrophied tergal glands situated under abdominal tergites VIII – X. The females are attractive to males and, upon direct contact, the two sexes form characteristic tandems. Chemical analyses indicated that the females secrete species-specific combinations of unbranched, unsaturated C_{12} primary alcohols from the tergal glands, (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (approx. 200 pg per female) and (3*Z*)-dodec-3-enol (185 pg) in *E. neotenicus*, (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (3500 pg) in *S. heyeri*, and (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (300 pg) and (3*Z*)-dodec-3-enol (50 pg) in *S. minutus*. (3*Z*,6*Z*,8*E*)-Dodeca-3,6,8-trien-1-ol and (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol act as major pheromone components in the respective species and mimic the function of female tergal gland extracts in electrophysiological and behavioral experiments. Biologically relevant amounts of the third compound, (3*Z*)-dodec-3-enol, elicited non-significant reactions in males of *E. neotenicus* and *S. minutus*, and slight synergistic effects in males of *S. minutus* when tested in combination with the major component.

Keywords *Silvestritermes minutus* · *Silvestritermes heyeri* · *Embiratermes neotenicus* · Sex-pairing pheromones · Tergal glands · Syntermitinae

Introduction

Independent colony foundation by alate dispersers, the primary reproductives, is by far the most frequent mode of reproduction of termite colonies. Only in a handful of cases has dependent colony foundation by wingless neotenic (secondary) reproductives accompanied by sterile helpers been documented, either as a result of budding or colony fragmentation. These rare cases are often associated with the propensity of colonization of new areas by invasive species (Evans et al. 2013; Vargo and Husseneder 2011). The dominance of colony reproduction by alate dispersers is surprising given that the capacity to produce neotenic reproductives is quite widespread in most basal termite taxa (Myles 1999). Moreover, low success rates are inherent to alate dispersal due to predation of alates, failure in mate search and low survival rates of incipient colonies (e.g. Lepage 1991). This contrasts with a great energy investment into alate production which can exceed one third of total colony biomass (e.g. Noirot and Darlington 2000; Thorne 1983).

In termites, the typical post-flight behavior of winged dispersers consists of spontaneous loss of wings, mate search and selection, and a nuptial promenade, during which the male follows the female until the tandem reaches a suitable site to

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establish a new colony (see Bagnères and Hanus 2015; Bordereau and Pasteels 2011; Nutting 1969). This behavioral sequence is mediated by a combination of long-range and short-range chemical signals. These include sex-pairing pheromones and contact chemoreception of non-volatile cues on the body surface of the partner. Sex-pairing pheromones are produced in most cases by females, with a few exceptions described in the basal isopteran families Archotermopsidae and Hodotermitidae, in which only males or both sexes produce the pheromones (Bordereau et al. 2010; Leuthold and Bruinsma 1978). Sex-pairing pheromones serve two complementary functions: male attraction over a relatively small radius, usually in the range of tens of centimeters, and prevention of accidental separation of the tandem by means of a trail laid by females of some species from their sternal glands during the nuptial promenade. The sex-pairing pheromones are secreted from the sternal glands, posterior sternal glands, tergal glands, or a combination of these exocrine organs of epidermal origin. Depending on the source of the pheromone, the females display characteristic calling behavior by exposing the intersegmental membranes covering the hypertrophied glands (Bordereau and Pasteels 2011).

The chemistry of sex-pairing pheromones has been studied in relatively few species across the phylogenetic diversity of termites and has revealed a conservative nature of termite pheromone communication in contrast with the great richness of defensive chemicals produced by termite soldiers. C_{13} or C_{14} branched aldehydes were identified as sex-pairing pheromones in the primitive family Archotermopsidae. In advanced clades, the chemistry of sex-pairing pheromones is dominated by unbranched and unsaturated C_{12} primary alcohols (3*Z*)-dodec-3-enol, (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol. They occur in all studied Rhinotermitidae and most Termitidae, except for the crown subfamily Nasutitermitinae, in which the isomers of diterpenes trinervitatriene and neocembrene were identified (reviewed in Bordereau and Pasteels 2011). In most cases, the sex-pairing pheromones were identified as single-component pheromones; however, the modern techniques used in the recent studies allowed detection of minor components in several species, suggesting that the sex-pairing pheromones may be more complex than previously thought (e.g. Bordereau et al. 2011; Wen et al. 2012, 2015).

The subfamily Syntermitinae is restricted to the neotropical region, contains over 100 species classified into 18 genera, and has recently been subjected to several taxonomic revisions (Rocha et al. 2012, 2017). Recent phylogenetic studies confirmed the monophyly of the group originally defined by the simultaneous presence of mandibles and frontal nasus in soldiers, but the internal cladogenesis at the generic level is still poorly resolved (Inward et al. 2007; Rocha et al. 2017). The chemistry of sex-pairing pheromones has been published for three sympatric species of the genus *Cornitermes*. C_{12} unsaturated alcohols were reported as major components, secreted from female tergal glands, and complemented by an additional volatile (*E*)-nerolidol, making up species-specific combinations (Bordereau et al. 2002, 2011). Unpublished observations, listed by Bordereau and Pasteels (2011), also propose a major role of the C_{12} alcohols in several other genera of Syntermitinae, sometimes in combination with (*E*)-nerolidol, or other sesquiterpenoids, and suggest that the tergal glands are the source of these compounds.

In the present paper, we report on the glandular origin and chemical identity of sex-pairing pheromones in three species of Syntermitinae occurring in sympatry in the rainforests of French Guiana, *Embiratermes neotenicus*, *Silvestritermes minutus* and *Silvestritermes heyeri* (formerly *S. holmgreni*), using histological studies, chemical analyses, behavioral observations and bioassays, and electrophysiological experiments. These three species are widely distributed in the Neotropics and use two very different breeding systems. While the colonies of *S. heyeri* appear to be exclusively headed by a pair of primary reproductives, most colonies of *E. neotenicus* and *S. minutus* contain one primary king accompanied by a harem of neotenic queens arising through parthenogenetic reproduction of a founding primary queen that replace her at some point of the colony life cycle (Fougeyrollas et al. 2015, 2017). Nevertheless, our direct observations and data on genetic structures of colonies and populations suggest that also in these two species, new colonies are exclusively founded by a pair of winged dispersers after the dispersal flight (Fougeyrollas et al. 2018).

Methods and Materials

Origin of Termites Colonies of the three studied species, containing last stage nymphs and alate dispersers, were collected in the rainforest of French Guiana along the route to Petit Saut (N5° 04.250′ W52° 58.770′ – N5° 04.650′ W53° 01.360′) prior to the dispersal period taking place in the mid or late phase of the principal rainy season (late April – July). Fragments of three colonies of *E. neotenicus* were collected in June 2016. One complete colony of *S. heyeri* and three complete colonies of *S. minutus* were collected in April 2017. The colonies or colony fragments were transported in plastic boxes to Prague and kept in glass aquariums on moist rainforest soil substrate (27 °C, 75% RH). Ready-to-fly alates emerged on colony surfaces in late April for *S. heyeri*, June and July for *S. minutus* and late July for *E. neotenicus*.

Histology of Exocrine Glands Dissected abdomens of imagoes (6–13 individuals) of both sexes in each of the species were fixed for 24 h at 4 °C in 2.5% glutaraldehyde (in 0.1 M cacodylate buffer at pH 7.2), stored in 1% formaldehyde or

5% glucose in 0.1 M cacodylate buffer and subsequently fixed in 1.5% osmium tetroxide for 22 h. The samples were then dehydrated in an ethanol series (30–100%) and propylene oxide. The specimens were embedded in Spurr resin (Spurr Low-Viscosity Embedding Kit, Sigma-Aldrich) and polymerized for 60 h at 62 °C. Semithin sections (1 μm) were prepared using Leica EM UC7, stained with toluidine blue and examined and photographed using a Carl Zeiss Amplival microscope equipped with a Canon EOS 600D camera.

Chemicals Synthetic standards of (3*Z*)-dodec-3-enol (DE) and (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE) were obtained as previously described (Jirošová et al. 2016), and (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE) was kindly supplied by Prof C. Bordereau from Université de Bourgogne in Dijon, France. The identity and purity of the standards were verified by means of two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC × GC-TOFMS) as described below (see also Electronic Supplementary Material). *n*-Hexane was redistilled prior to use.

Solvent Extracts and Solid Phase Microextraction (SPME) Solvent extracts of three origins were used in different experiments: female tergal gland extract (TGE), female sternal gland extract (SGE) and whole body extract (WBE). Tergal regions containing the tergal glands (tergites VIII – X) and the sternal region (including the sternal gland at sternite V) were dissected from cold anesthetized female alates. Prior to dissection, the females were allowed to fly in empty glass jars until they spontaneously shed their wings and started the calling behavior. The tissues were dissected under a stereomicroscope and extracted in hexane (5–10 μl per individual) for 15 min under sonication. WBE was prepared using 12 h extraction at 4 °C without sonication. For identification and quantification purposes, several solvent extracts were prepared independently from 15 to 50 individuals originating from all available colonies of each species. For electrophysiology and behavioral experiments, extracts from the same colony as the tested termites were always used.

Body surface sampling was done with Supelco DVB/CAR/PDMS (grey) SPME fibers (50 μm film thickness; Merck, Darmstadt, Germany). Living imagoes were gently immobilized using soft forceps and rubbed with the fiber on the tergal region covering the tergal glands, the sternal region covering the sternal gland and/or on the control anterior tergal surface distant from the tergal glands. For each type of sample, 7 individuals were used, and each was rubbed 200 times in a given region. The fiber was immediately inserted into the injection port of a gas chromatograph and desorbed for 1 min.

Gas Chromatography Solvent extracts and SPME extracts were first analyzed on a gas chromatograph 7890 N (Agilent Technologies, Santa Clara, CA, U.S.A.), equipped with a polar DB-WAX column (30 m, id 0.25 mm, 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.A.), connected to an Agilent 5975C mass-selective detector (70 eV electron ionization, source temperature 230 °C). The transfer line was heated to 250 °C. The column temperature was held at 50 °C for 1 min, increased to 150 °C at 5 °C/min and then at 10 °C/min up to 250 °C, and held for 5 min. Helium was used as carrier gas at 1 ml/min flow rate. The split/splitless injection port was heated to 200 °C, and samples were injected in splitless mode with a purge time of 1 min.

Detailed analyses and quantifications were performed using a two-dimensional gas chromatograph with time-of-flight mass spectrometric detection (GC × GC-TOFMS; Pegasus 3D, Leco, St. Joseph, MI, U.S.A.), equipped with a combination of non-polar ZB-5MS (30 m, id 0.25 mm, 0.25 μm film thickness; Phenomenex, Torrance, CA, U.S.A.) and medium polarity RTX-50 (1.5 m, id 0.1 mm, 0.1 μm film thickness; Restek, Bellefonte, PA, U.S.A.) columns. The split/splitless injection port was heated to 250 °C. The temperature program for the primary column was 50 °C (1 min) to 320 °C (20 min) at 8 °C/min, and the secondary column was set 10 °C higher.

Chemical identifications were based on comparisons of MS fragmentation patterns and Kovats indices of detected analytes with those of synthetic standards, reported in the literature or obtained in our previous studies. To estimate the quantity of DE, DDE and DTE in extracts, calibration curves were constructed using 10, 100 and 1000 pg/μl solutions of synthetic standards of the three compounds.

Electrophysiology To evaluate the perception of individual compounds from female tergal glands by male alates, we performed a series of experiments using dose-response electroantennography (EAG) and gas chromatography coupled with electroantennographic detection (GC-EAD). The electrophysiological setup is described in detail in Hanus et al. (2009).

For GC-EAD analyses, 5890A Hewlett-Packard gas chromatograph (Agilent) was equipped with a DB-WAX column (30 m × 0.25 mm × 0.25 μm; J&W Scientific). The GC was operated at an initial temperature of 40 °C for 2 min, then ramped to 240 °C at a rate of 8 °C/min and the final temperature was held for 10 min. The temperatures of the GC inlet and detector were set to 250 °C and 270 °C, respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. GC-EAD experiments were performed with TGE and solutions of standards of candidate pheromone components of each species (DTE, DDE, and DE or their mixtures). Some of analyzed solutions were co-injected with a series of *n*-alkane standards ($C_8 – C_{22}$) to estimate the retention indices of analytes eliciting systematic antennal responses. The region of antennal activity was considered to be established when the

EAD response was observed in at least three independent experiments at identical retention times.

EAG dose-response experiments were performed with males of *E. neotenicus* and *S. minutus* to assess the relative importance of each of the two candidate pheromone components identified in these species, to determine their quantity in tergal glands of females and to test for possible synergistic effects of their mixtures. We tested series of 1 pg – 10 ng of standards of the candidate pheromone components DE, DDE and DTE, or their mixture, and compared their effect with that of 0.5 and 1 female equivalent (Eq) of TGE per treatment. Hexane was used as negative control and all responses were normalized to air stimulations, performed before and after each series. Each stimulation series was repeated with six to ten different males and the data was evaluated using analysis of variance (ANOVA), followed by the least square difference (LSD) post-hoc comparison test. Prior to the calculations, the normality of the data was tested using Shapiro-Wilk test and the homogeneity of variances assessed using Levene's test.

Courtship Behavior and Sex Attraction Bioassays Behavioral experiments were conducted at room temperature (23 °C) under artificial light. Prior to the experiments, alates emerging from the colonies were allowed to fly in empty glass jars and spontaneously shed their wings.

To test the readiness of imagoes to pair, dealates of both sexes were introduced in a Petri dish lined with filter paper and observed with respect to the behavioral repertoire specific to dealates, especially mate search, calling behavior of females and tandem formation.

Three experimental designs were used in subsequent sex attraction bioassays: the mid-range non-contact bioassay, the short-range bioassay, and the T-maze trail-following bioassay, described below and in the Electronic Supplementary Material (Figs S1 – S3).

Mid-range experiments (Fig. S1) were designed to test the attractiveness of airborne volatiles from calling females or of TGE and SGE to males (eventually to females) without direct contact with the stimulus. One calling female and one male, or square pieces of filter paper treated with TGE or SGE were introduced separately into glass cylinders (height 2 cm and Ø 3 cm for *E. neotenicus* and *S. heyeri*, height 1.3 cm and Ø 2.3 cm for *S. minutus*) placed on a glass Petri dish lid either 3 cm or 4.5 cm away from one another. One pair of termites or treated papers was used for 5 repetitions of the experiment with 5 different focal dealates. The cylinders were covered with chrome nickel steel mesh (wire Ø 0.12 mm, mesh Ø 0.198 mm). Between different experiments, all parts of the setup were washed 3 times with analytical grade ethanol and boiling distilled water and then heated to 300 °C for 30 min. One male (or in one experiment one female) dealate was placed on the mesh and the mesh covered with another glass Petri dish lid. Behavior of the focal animal was recorded for 300 s, the recordings were analyzed using JWatcherVideo software, and the time the termites spent above each of the cylinders scored. We calculated the Preference Index as $PI = (t_1 - t_2)/(t_1 + t_2)$, where t_1 and t_2 stand for the time spent above individual cylinders (Williams and Mendelson 2011). The PI data was arcsine transformed using the formula arcsine $\sqrt{((PI + 1) / 2)}$ and tested for normality using the Shapiro-Wilk test. 95% Confidence intervals were calculated from the distribution of the transformed PIs fitted to the t-distribution and compared with the value expected under no preference.

Short-range experiments (Fig. S2) tested the attractiveness of TGE, SGE, WBE and serial dilutions of candidate compounds DE, DDE, and DTE over short distances and allowed males to touch the object emitting the chemical stimuli. One male dealate was introduced into a 9 cm (*E. neotenicus*, *S. heyeri*) or 6 cm (*S. minutus*) plastic Petri dish lined with dry Whatman No. 1 filter paper. A new Petri dish was used for each experiment to avoid contamination. Folded pieces of filter paper (1 × 1.5 cm for *E. neotenicus* and *S. heyeri* or 0.8 × 1.3 cm for *S. minutus*) were placed in opposite halves of the Petri dish and treated with 2.5–10 μl of the tested solutions. The solvents were left to evaporate for 30 s. TGE, WBE and SGE, and serial dilutions of candidate compounds DE, DDE, DTE or their mixtures were tested against a hexane control or in various combinations. Behavior of the males was recorded for 300 s and the recordings analyzed using JWatcherVideo software. Time spent by the males in the radius around the papers (Ø 3.5 cm for *E. neotenicus* and *S. heyeri* or 2.3 cm for *S. minutus*) or in contact with the papers was used to calculate the Preference Index, which was further transformed and statistically analyzed as described above for the mid-range bioassays. Each experiment was performed with 10–30 different males. Males that did not change their behavioral status for more than 270 s were discarded from the calculations as inactive.

The T-maze experiment (Fig. S3) was used as a second type of short-range bioassay, testing the contact perception and preference of males for different chemical stimuli. The arena was made from a 0.6 cm Plexiglas plate with a 3 cm entrance corridor and 7 cm long branches (0.3 × 0.3 cm). One or two μl/cm of tested stimuli were applied on filter paper under the entrance corridor and one of the branches (3 + 7 cm). Individual males entered the maze through the entrance corridor from a small round arena. The choice of individual branches was scored for 30–90 different males and the data was analyzed using the χ^2 test.

Results

Courtship Behavior and Source of the Sex-Pairing Pheromone Freshly and spontaneously dealated females of all three

studied species, kept in female-only groups, alternated in short intervals the exploration or escape behaviors with calling behavior, during which they stood for several seconds in the characteristic calling posture with raised abdomen. Calling females of all three species conspicuously exposed the intersegmental membranes in the tergal region of the posterior abdomen, suggesting that the sex-pairing pheromone is produced in the tergal glands (Fig. 1). In groups containing both sexes, the males of the three species performed typical searching and pairing behavior; they ran quickly across the Petri dish, waved their antennae, and as soon as they approached a female formed a tandem, with their antennae and mouthparts touching the tergal region of the female's abdomen (Fig. 1). In male-only groups, the males eventually formed tandems with other males, a behavior not observed in females.

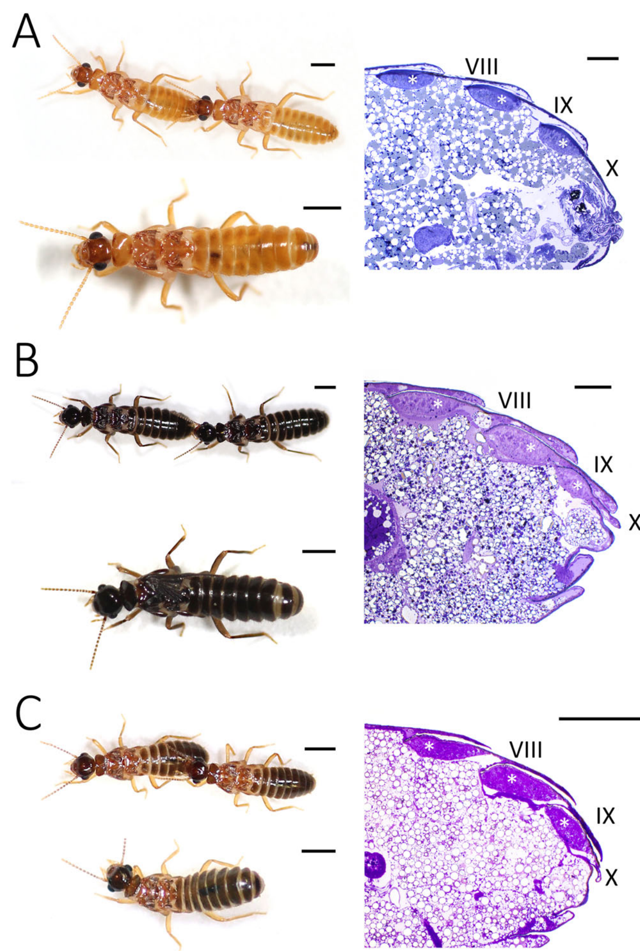

Fig. 1 Courtship behavior and source of sex-pairing pheromone in *Embiratermes neotenicus* (**a**), *Silvestritermes heyeri* (**b**) and *Silvestritermes minutus* (**c**). Photographs on the left show tandems of dispersing imagoes (male follows the female), and female imagoes in a calling posture with exposed tergal glands. Scale bars represent 1 mm. Photographs on the right show sagittal sections of the posterior abdomen of female imagoes. Asterisks mark the tergal glands situated in anterior parts of abdominal tergites VIII, IX and X. Scale bars represent 0.1 mm

Figure 1 shows the sections of the posterior abdomens of female dealates and, consistent with behavioral observations, suggest that the tergal glands are the major source of sex-pairing pheromones. In all three species, the tergal glands are situated under the tergites VIII – X and are well developed. In contrast, we did not observe a hypertrophied sternal gland, another possible source of sex-pairing pheromones, or other abdominal exocrine organ in any of the species.

Chemistry of Female Tergal Glands Both the TGE and SPME samples of the female tergal region in *E. neotenicus* revealed the presence of two conspicuous peaks, consistently occurring in TGE of females from all three colonies while being absent in the control SPME samples of the anterior abdominal tergites (Fig. 2). In trace amounts, the two compounds were also detected in SPME samples of the sternal region. Accordingly, the two compounds were identified in WBE of females and were absent from the WBE of male dealates. Based on their Kovats retention indices (KI 1459 and KI 1496) and characteristic fragmentation patterns (diagnostic fragment ions *m/z* 166, 81, 68 and 180, 91, 79), the compounds were identified as DE and DTE, respectively. The identity of the compounds was confirmed by injections of synthetic standards of DE and DTE, which matched the mass spectra and retention times of the candidate analytes (Fig. S4). GC × GC-TOFMS quantification of four independent TGE estimated the amount of DE and DTE in one female to be 185 ± 55 pg and 209 ± 108 pg, respectively (mean ± standard deviation)(Table 1).

TGE as well as SPME sampling of the tergal gland region in *S. heyeri* females indicated a single prominent peak specific to tergal glands. This peak was absent from all control samples, including the SGE and SPME samples from the sternal region (Fig. 3). This compound was detected in WBE of females but absent in WBE of male imagoes. The retention index of the compound (KI 1453) differed from those of DE and DTE, but matched that of DDE, as well as its fragmentation pattern with diagnostic fragment ions *m/z* 164, 79, and 67. This identification was confirmed by injection of synthetic DDE (Fig. S4). The quantity of DDE in tergal glands of one female was estimated to be 3603 ± 1080 pg based on GC × GC-TOFMS analyses of three independently prepared TGE (Table 1).

The small body size of *S. minutus* females precluded reliable SPME analysis. Hexane extraction revealed the presence of two consistently occurring peaks unique to TGE when compared to control extracts, i.e. female SGE and male WBE (Fig. 4). Both peaks were also detected in female WBE. They were identified as DDE and DE based on the match of both the retention indices (KI 1453 and KI 1459, respectively) and fragmentation patterns (Fig. S4). Three independent TGE allowed us to estimate the quantities of DDE and DE in one female tergal glands to be 318 ± 91 pg and 53 ± 14 pg, respectively (Table 1).

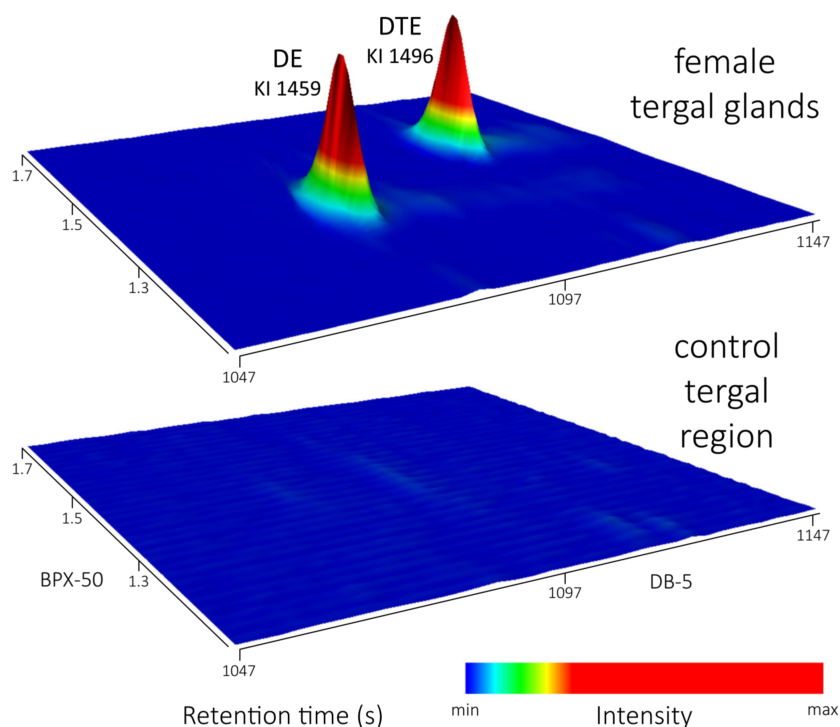

Fig. 2 Characteristic GC × GC chromatograms of volatiles from *Embiratermes neotenicus* female alates obtained by SPME sampling of tergal gland region (top) and control tergal region at anterior abdominal tergites, remote from the tergal glands (bottom). The chromatograms show selected retention window in which two analytes specific to female tergal glands, i.e. DE and DTE, were reproducibly detected

GC-EAD Analyses GC-EAD experiments with males of *E. neotenicus* and female TGE indicated a strong antennal response at the retention time corresponding to that of DTE (Fig. 5a). In contrast, in spite of an equivalent amount of DE detected in the TGE, the antennal responses corresponding to this compound were low or absent. This observation was confirmed by GC-EAD experiments with synthetic standards of both compounds. While DTE elicited strong and consistent antennal responses over a range of biologically relevant concentrations, only an overdose of DE resulted in discernible responses.

GC-EAD recordings from antennae of *S. heyeri* males using conspecific female TGE indicated a consistent and strong response at the retention time corresponding to DDE (Fig. 5b). The sensitivity of males to the compound was confirmed by injection of synthetic DDE.

In GC-EAD recordings from antennae of *S. minutus* males, there were two consistent responses to female TGE at retention times matching those of the candidate compounds specific to female tergal glands, DE and DDE (Fig. 5c). The response to DE was always lower than that to DDE. The same pattern was observed when synthetic compounds were co-injected, and only an increase in the proportion of DE in the tested blend from the natural ratio of DE:DDE ≈ 1:10 to 1:1 elicited equal responses to both compounds.

Table 1 Quantity of pheromone components in female tergal gland extracts estimated using GC × GC-TOFMS

Species	N	Compound	Quantity per individual (pg) mean ± SD
Embiratermes neotenicus	4	DE	185 ± 55
		DTE	209 ± 108
Silvestritermes heyeri	3	DDE	3603 ± 1080
Silvestritermes minutus	3	DE	53 ± 14
		DDE	318 ± 91

EAG Tests Results of dose-response experiments with antennae of male *E. neotenicus* and TGE and serial dilutions of DE, DTE and their 1:1 mixture corresponding to the natural ratio (Fig. 6a), were consistent with the sensitivity patterns observed in GC-EAD analyses. DTE elicited a strong and dose-dependent response in biologically relevant concentrations, with the response to 100–1000 pg similar to that to 1 female equivalent of TGE, corresponding approximately to the GC quantification of DTE (209 pg). In contrast, the quantity of DE needed to give an equivalent response to that to TGE was an order of magnitude higher than that estimated to be present in TGE (185 pg). When the blend of DE and DTE in the natural ratio (≈ 1:1) was tested, no difference was observed in the responses of male antennae when compared to DTE tested alone. These results confirm the weak perception of DE by male alates, in agreement with the data obtained by GC-EAD, and at most a small synergistic effect of the mixture of the two compounds.

EAG recordings from antennae of male *S. minutus* (Fig. 6b) confirmed a strong sensitivity to DDE which gave a similar response to that to the TGE at dosages of 100–1000 pg, corresponding to the GC quantification of 318 pg of DDE per female. DE showed only weak electroantennographic activity

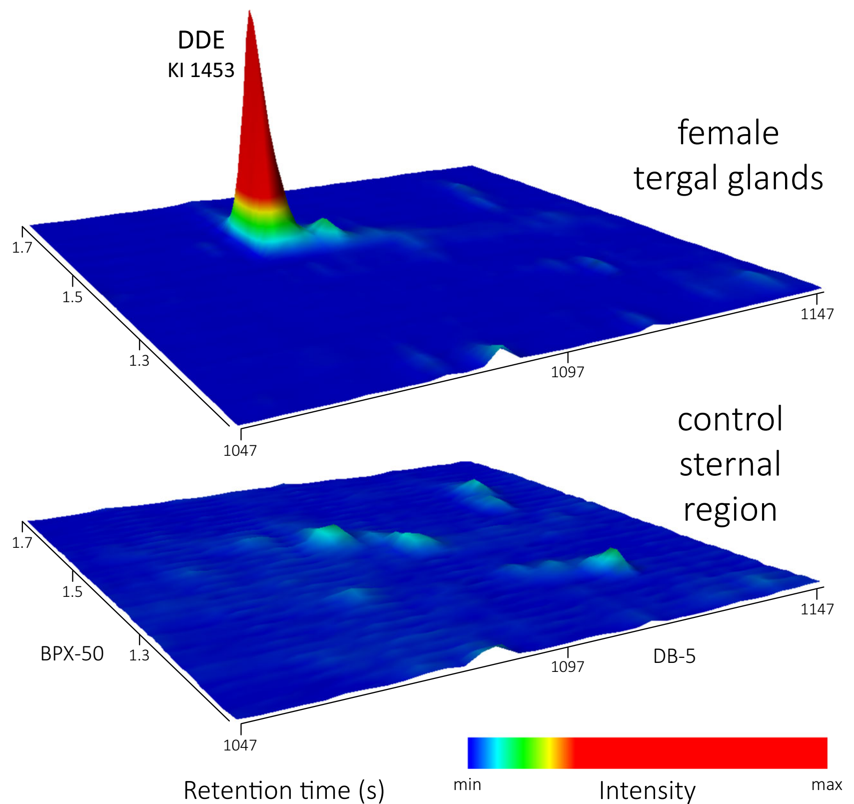

Fig. 3 Characteristic GC × GC chromatograms of volatiles from *Silvestritermes heyeri* female alates obtained by SPME sampling of tergal gland region (top) and control sternal region of abdomen including the sternal gland area (bottom). The chromatograms show a selected retention window in which the analyte specific to female tergal glands (DDE) was reproducibly detected

with 1000 pg required to give a response equal to that of 0.5 female equivalent, the response to the latter being mainly due to DDE as the amount of DE in the TGE was only 53 pg/female. The mixture of DDE and DE in the natural ratio (≈ 10:1) indicated a slight synergistic effect of the two compounds; responses to the mixture outcompeted those to TGE in doses by one order of magnitude lower than the responses to DDE alone.

Sex Attraction Bioassays Mid-range experiments with *E. neotenicus* males confirmed attractiveness of airborne volatiles from live females to males, contrasting with indifferent

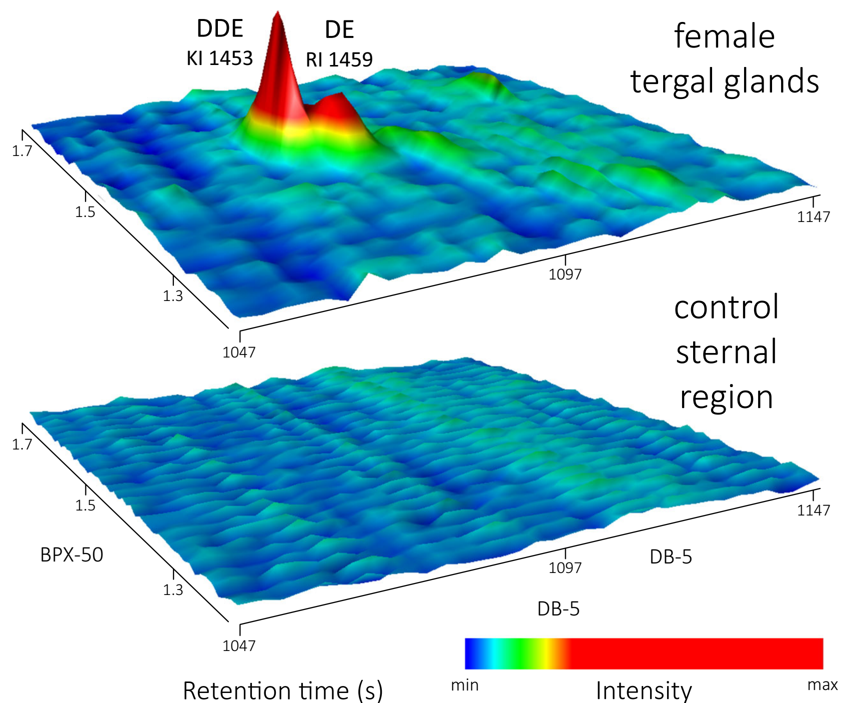

Fig. 4 Characteristic GC × GC chromatograms of volatiles from *Silvestritermes minutus* female alates obtained by hexane extraction of dissected tergal glands (top) and dissected sternal epidermis from the abdomen including the sternal gland (bottom). The chromatograms show a selected retention window in which two analytes specific to female tergal glands (DDE and DE) were systematically detected

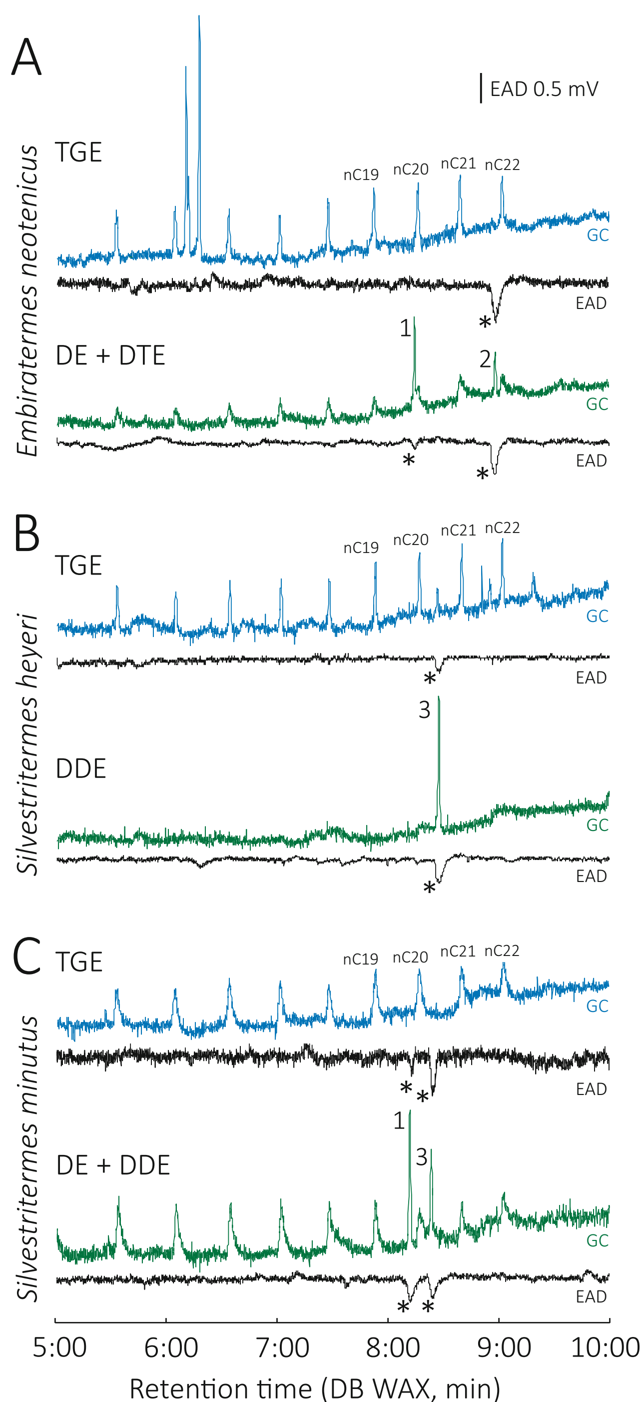

Fig. 5 Antennal responses from males in GC-EAD experiments. Antennae of males were exposed to tergal gland extracts (TGE) from females or synthetic standards of candidate compounds DE (**1**), DTE (**2**), or DDE (**3**), co-injected with *n*-alkane standards (C_8 – C_{22}) on a DB-WAX column. **a** *Embiratermes neotenicus*. **b** *Silvestritermes heyeri*. **c** *Silvestritermes minutus*. Asterisks mark systematically occurring antennal responses. nC_{19} – nC_{22}, chromatographic peaks of alkane standards

reactions of females to dealates of both sexes (Fig. 7a). In contact short-range experiments, the males showed a high preference for female WBE and TGE when compared to the controls, as well as a preference for TGE in comparison to SGE (Fig. 7b, c). Activity threshold in experiments with serial dilutions of DTE was a dose of 10 pg; all doses above 1 pg were significantly preferred by males (Fig. 7d). When compared with 0.5 female equivalent of TGE, the dose of 1000 pg DTE was significantly more attractive, unlike the lower doses. DE was relatively unattractive with activity threshold at 100 pg, and none of the tested doses (up to 1000 pg) were as attractive as the TGE (Fig. 7e). Both tested concentrations of the mix of DTE and DE in the natural 1:1 ratio elicited a small but non-significant preference when compared to the same dose of DTE alone (Fig. 7f).

Trail-following T-maze experiments showed similar trends to those observed in short-range arena assays, yet differed in some quantitative aspects (Fig. 7g). The males clearly preferred TGE to SGE. Higher concentrations of DTE were needed to outcompete TGE when compared to the short-range assay: only at the highest dose of 1000 pg was the synthetic compound selected significantly more often by males than 0.1 female equivalent of TGE. At all tested doses of DE, males followed the trail made of TGE significantly more often. As for DTE alone, the 1:1 mixture with DE was also more attractive than 0.1 female equivalent of TGE only at the highest dose. In agreement with observations from the arena assay and the EAG data, there was no significant synergistic effect of a DTE:DE 1:1 mixture when compared to DTE alone.

Males of *S. heyeri* clearly preferred female to male dealates in the mid-range experiment, as well as female WBE and TGE when compared to the hexane control in the short-range experiment (Fig. 8a, b). The sensitivity threshold for the single candidate pheromone compound, DDE, was determined to be 100 pg in an experiment performed with serial dilutions of the standard.

Male dealates of *S. minutus* significantly preferred TGE to SGE in mid-range experiments as well as TGE to hexane in short-range assays (Fig. 9a, b). Short-range experiments indicated the sensitivity threshold of males at a dose of 10 pg of DDE. At doses of 100 and 1000 pg, DDE was equally attractive to males as was 0.1 female equivalent of TGE (Fig. 9c). In contrast, none of the tested doses of DE were preferred over hexane and TGE was always significantly more attractive to males than any of the tested DE dilutions (Fig. 9d). A mixture of DDE:DE in the ratio of 10:1, which is approximately equal to the ratio of these compounds in TGE, was as attractive to males at doses of 100:10 and 1000:100 pg (DDE:DE) as was 0.1 female equivalent of TGE (Fig. 9e). When the DDE:DE mixture was tested against equivalent doses of DDE, a marginal, non-significant preference for the mixture at a dose of 10:1 pg DDE:DE vs. 10 pg DDE and a marginal, but significant preference for the mixture at 100:10 pg DDE:DE vs. 100 pg DDE was observed. In T-maze experiments, TGE was always preferred to SGE (Fig. 9f). A dose of 100 pg of DDE was selected as often as 0.1 female equivalent of TGE,

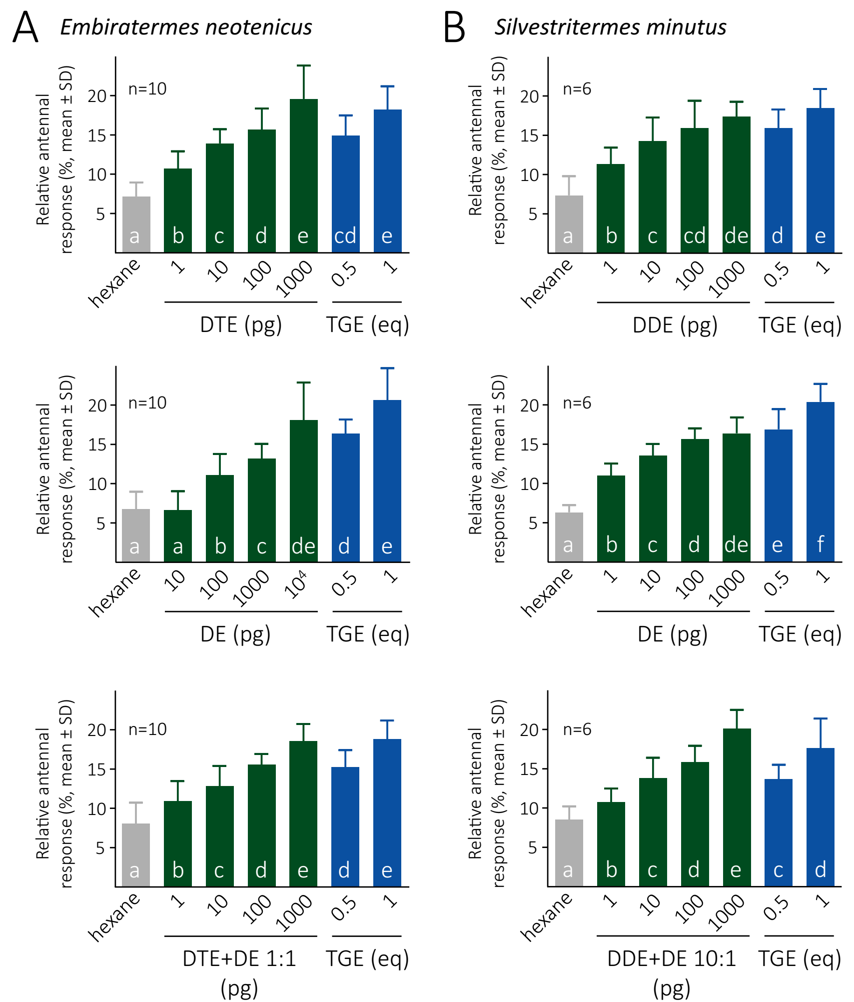

Fig. 6 Antennal responses of males in dose-response EAG experiments with *Embiratermes neotenicus* (**a**) and *Silvestritermes minutus* (**b**). Antennae were exposed to hexane, serial dilutions of candidate pheromone components DE, DDE, DTE and their mixture and 0.5 and 1 equivalent (Eq) of female tergal gland extracts (TGE). The data are based on a series of stimulations of ten and six different males in *E. neotenicus* and *S. minutus*, respectively. Columns marked with the same letter do not significantly differ at $\alpha = 0.05$ (ANOVA followed by LSD post-hoc comparison)

and 1000 pg of DDE was significantly more attractive than 0.1 female equivalent of TGE. In contrast, 0.1 female equivalent of TGE was always selected more often by males than any of the doses of DE. A non-significant preference was observed between 0.1 female equivalent of TGE and 100:10 pg mixture of DDE:DE. At a higher dose, the mixture was significantly preferred. Finally, non-significant differences were observed in the comparison of 10 pg DDE vs. 10:1 pg DDE:DE and 100 pg DDE vs. 100:10 pg DDE:DE.

Discussion

We studied the chemistry of sex-pairing pheromones in alate dispersers of three species from the subfamily Syntermitinae, *Embiratermes neotenicus*, *Silvestritermes heyeri* and *Silvestritermes minutus*. These species are widespread in the Neotropics and particularly abundant in the rainforests of French Guiana. Females of the three species possess hypertrophied tergal glands situated under abdominal tergites VIII – X. After dispersal and the spontaneous loss of wings, females expose the tergal glands. This is attractive to males and results in formation of characteristic tandems. Female tergal glands secrete species-specific combinations of C_{12} alcohols: (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (DTE, approx. 200 pg in one female) and (3*Z*)-dodec-3-enol (DE, 185 pg) in *E. neotenicus*, (3*Z*,6*Z*)-dodeca-3,6-dien-1-ol (DDE, 3500 pg) in *S. heyeri*, and DDE (300 pg) and DE (50 pg) in *S. minutus*. While DTE and DDE clearly act as major components of the sex-pairing pheromones in the respective species and mimic the biological activity of female tergal gland extracts, the third compound, DE, elicited non-significant reactions in males of *E. neotenicus* and *S. minutus* in biologically relevant amounts. A marginally significant synergistic effect of DE has only been recorded in *S. minutus* males when tested in combination with the major component DDE.

The topology of female tergal glands in the three species is similar to that observed in other species, including the syntermitine genus *Cornitermes* (Ampion and Quennedey 1981; Bordereau et al. 2002). The sternal glands did not show evidence of hypertrophy, suggesting that the tergal glands are the dominant or only pheromone source, as is also the case in

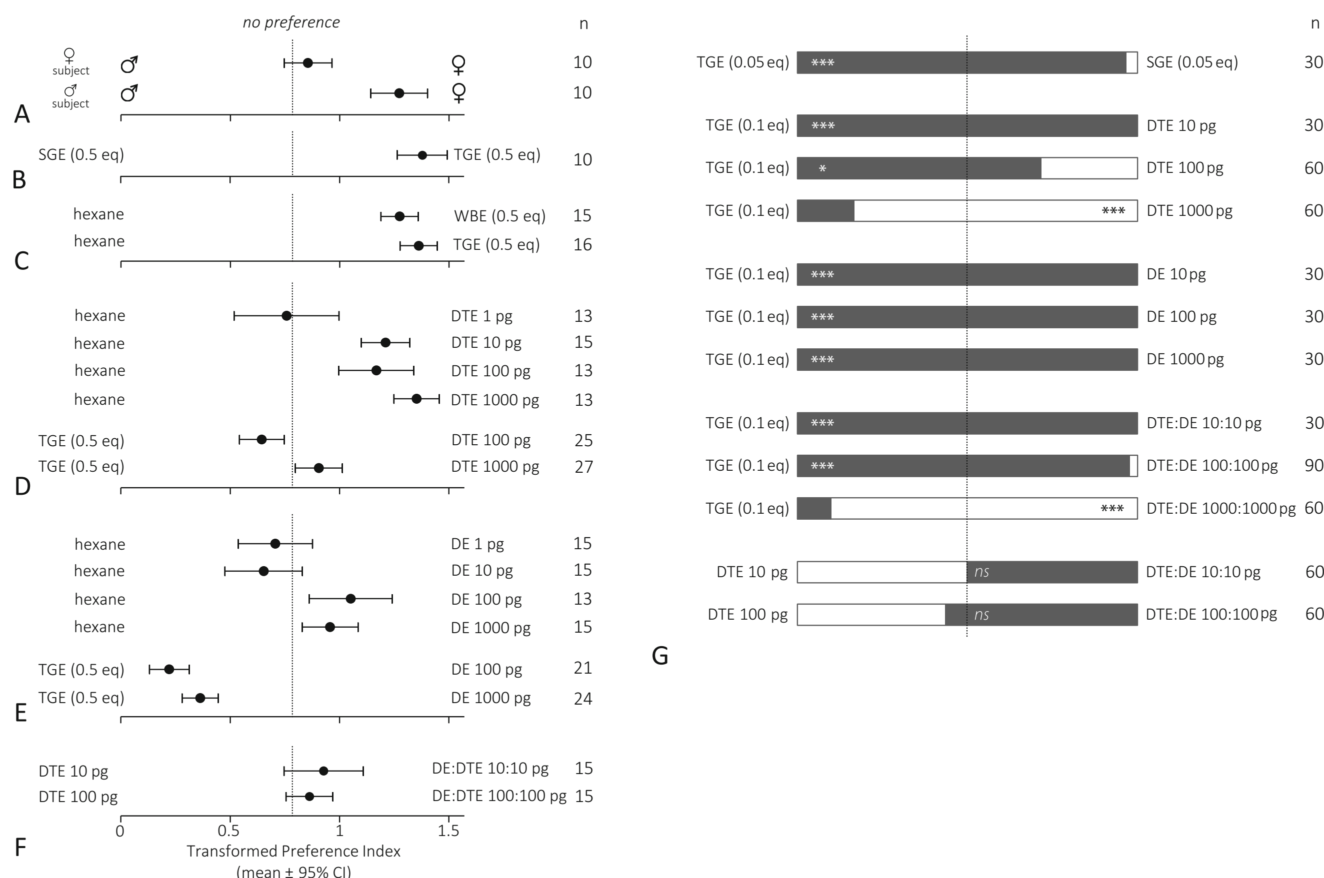

Fig. 7 Behavioral bioassays with imagoes of *Embiratermes neotenicus*. **a** Mid-range bioassay with living male and female imagoes as stimuli and males and females as subjects. **b** Mid-range bioassay with female sternal gland extract (SGE) and female tergal gland extract (TGE) and males as subjects. **c** Short-range bioassays with whole body extract (WBE) and TGE offered to one male imago on folded pieces of filter paper as compared to a solvent control. **d**, **e** Short-range bioassays with serial dilutions of DTE and DE, tested against solvent control or TGE. **f** Short-range bioassays with serial dilutions of DTE versus a 1:1 mixture of DTE:DE. The data show means ±95% confidence intervals, calculated from a transformed Preference Index. **g** T-maze bioassays comparing the preferences for TGE vs. SGE, TGE vs. serial dilutions of DTE, DE and their mixture, and DTE vs. DTE:DE

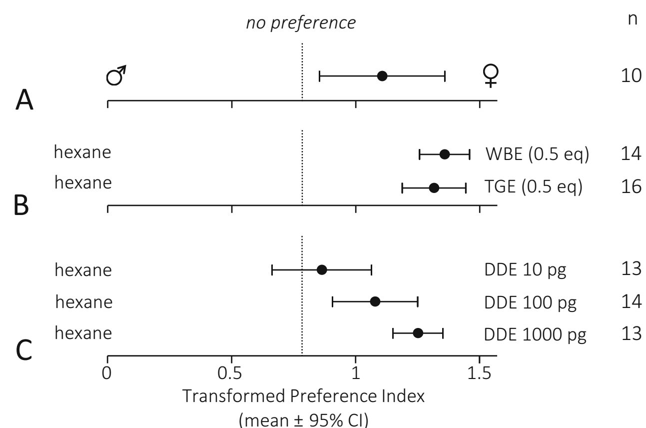

Fig. 8 Behavioral bioassays with imagoes of *Silvestritermes heyeri*. **a** Mid-range bioassay with living male and female imagoes as stimuli and males as subjects. **b** Short-range bioassays with whole body extract (WBE) and female tergal gland extract (TGE) offered to one male imago on folded pieces of filter paper compared to a solvent control. **c** Short-range bioassays with serial dilutions of DDE compared to a solvent control. The data shows means ±95% confidence intervals, calculated from a transformed Preference Index

Cornitermes (Bordereau et al. 2002, 2011). Only in *E. neotenicus* did we also detect DTE and DE in SPME samples of the sternal glands. However, their quantity and attractiveness of the sternal gland to males appear negligible (Fig. 7b, c). Nevertheless, we cannot exclude the possibility that the compounds are secreted from the sternal gland as a species-specific pheromone trail during the nuptial promenade since simultaneous secretion of the pheromone by both glands has been previously documented (see Bordereau et al. 2011; Sillam-Dussès et al. 2011).

Even though the biological activity of DE was very low in both *E. neotenicus* and *S. minutus*, a marginally significant additive effect of the mixture DDE + DE was observed in *S. minutus* (Fig. 9e). Secretion of DE by the tergal glands of females has been documented in the syntermitines *Cornitermes silvestrii* (Bordereau et al. 2011), *Cornitermes snyderi*, *Embiratermes festivellus* and *Syntermes praecellens* (all three reported as unpublished observations in Bordereau and Pasteels 2011) and by the sternal gland in the

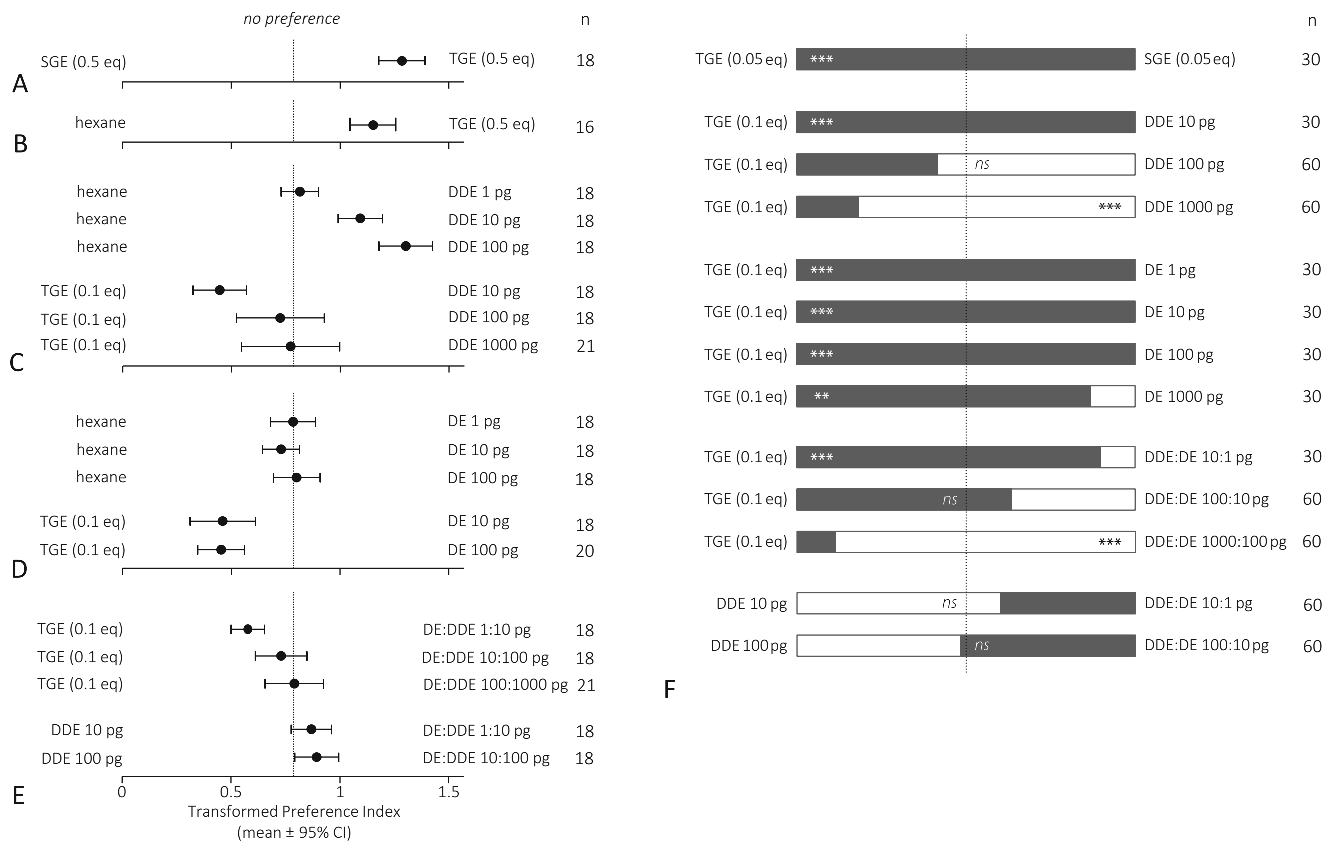

Fig. 9 Behavioral bioassays with imagoes of *Silvestritermes minutus*. **a** Mid-range bioassay with female sternal gland extract (SGE) and female tergal gland extract (TGE) and males as subjects. **b** Short-range bioassays with TGE offered to one male imago on folded pieces of filter paper compared to a solvent control. **c**, **d** Short-range bioassays with serial dilutions of DDE and DE, tested against a solvent control or TGE. **e** Short-range bioassays with serial dilutions of a 10:1 mixture of DDE:DE versus TGE or DDE. The data shows means ±95% confidence intervals, calculated from a transformed Preference Index. **f** T-maze bioassays comparing the preferences for TGE vs. SGE, TGE vs. serial dilutions of DDE, DE and their mixture, and DDE vs. DDE:DE

macrotermitines *Ancistrotermes dimorphus* (Wen et al. 2015) and *Odontotermes formosanus* (Wen et al. 2012). Behavioral roles of DE are controversial. Long-range attraction of males was suggested in *O. formosanus* (Wen et al. 2012), while no effect on attraction or species-specific female discrimination was reported in *C. silvestrii* (Bordereau et al. 2011), and *A. dimorphus* (Wen et al. 2015). Even though our results indicate an eventual role of the compound as a minor component in the pheromone of *S. minutus*, the example of *E. neotenicus* adds to the doubts about its biological significance. Alternatively, the presence of DE may be interpreted as a non-specific by-product of the biosynthesis of the major pheromone components. The most likely biosynthetic origin of the unsaturated C_{12} alcohols consists of three cycles of ß-oxidation of oleate or linoleate followed by reduction of the carboxyl moiety, giving rise to DE and DDE, respectively, and a subsequent stereospecific desaturation at the omega-4 position leading to DTE. Thus, the presence of DE in tergal glands can be the result of catabolism analogous to that of DDE or DTE being applied to oleate, which is abundant in the fat bodies of insects, including termites (e.g. Šobotník et al. 2006). However, it should be noted that DE is the genuine trail-following pheromone in all Kalotermitidae and many Macrotermitinae.

Given the low responses of males to DE, the quantities of the major components DTE in *E. neotenicus* and DDE in *S. minutus* that are needed to mimic the effect of tergal gland extracts were expected to be approximately equal to those in the extracts. Indeed, dose-response EAG and short-range behavioral assays indicated that quantities of the two compounds needed to compete with the activity of conspecific tergal gland extracts were similar to their average content in the glands. There were some differences between the short-range experiments and the T-maze contact experiments. Tergal gland extracts in the latter experiment (Figs. 7g and 9f) elicited a stronger preference in males then expected from the GC quantification and short range bioassays. We speculate that these differences were due to additional compounds of low volatility in the tergal gland extract, such as cuticular hydrocarbons and lipids from the fat body, that can be detected by the antennae of males in the T-maze.

Our observations, together with those on other Syntermitinae, support the dominance of the unsaturated C_{12} alcohols in sex-pairing communication in most lineages of Neoisoptera (Bordereau and Pasteels 2011). We may expect that DDE and DTE, and possibly DE, will be components in sex-pairing pheromones of other Syntermitinae. The apparent species-specificity of the sex-pairing pheromones in the three sympatric species studied here becomes less obvious when we consider the weak or negligible pheromone role of DE. Nevertheless, the three species differ in the major component either qualitatively (DTE vs. DDE) or quantitatively (DDE in *S. heyeri* and *S. minutus*). We have little data on the time of day at which dispersal flights occur in the three species. However, our observations on the maturation and occurrence of flying alates in both field and laboratory-held colonies suggest a shift in the dispersal seasons, taking place in late April and early May in *S. heyeri*, in June and July in *S. minutus*, and in late July in *E. neotenicus*. A temporal separation of dispersal has been proposed as the major mechanism of reproductive isolation in a number of sympatric situations. In some cases, however, even the restricted chemical repertoire of termite sex-pairing pheromones was shown to be sufficient to mediate preference of conspecific mates over related species with simultaneous timing of dispersal, either due to a different single-component pheromone or species-specific minor components (e.g. Peppuy et al. 2004, Bordereau et al. 2011, reviewed in Bordereau and Pasteels 2011).

Available phylogenetic hypotheses point to important gaps in definitions of several syntermitine genera. This also applies to the species-rich genus *Embiratermes*, which splits over multiple internal clades within the monophyletic subfamily (Rocha et al. 2017). The most recent topology confirms a close relationship between *E. neotenicus* and *E. festivellus* as well as between *Silvestritermes minutus* and *S. heyeri* (Rocha et al. 2017). Each pair of species possesses a common major sex-pairing pheromone component, i.e. DTE in *Embiratermes* (see unpublished data reported for *E. festivellus*; Bordereau and Pasteels 2011) and DDE in *Silvestritermes*. This suggests conservation of the major pheromone component at low phylogenetic levels.

Bordereau et al. (2011) proposed an additional component in the sex-pairing pheromone blend in some *Cornitermes* species, the sesquiterpene alcohol (*E*)-nerolidol. When tested alone, the compound did not show any behavioral activity, but it enhanced the attractiveness of conspecific females for the males when tested in mixture with the major component. The existence of a sesquiterpenoid minor component was also proposed in *Embiratermes festivellus* (Bordereau and Pasteels 2011). However, our observations in the three syntermitine species, including the genus *Embiratermes*, did not confirm the presence of (*E*)-nerolidol or other sesquiterpenoids in the tergal glands of dispersing females. In contrast, during our survey of volatiles produced by fertile and physogastric primary and secondary queens in several Syntermitinae, we identified enantiomerically pure (3*R*)-(*E*)-nerolidol in large amounts in the headspace of queens in *Embiratermes neotenicus*, *Labiotermes labralis*, and *Silvestritermes heyeri* (unpublished observations). Moreover, this compound has also been detected in large quantities in the hemolymph, fat body and ovaries of maturing and mature queens. Behavioral and electrophysiological experiments did not reveal evidence of perception of the compound by nestmates. The large amounts of (3*R*)-(*E*)-nerolidol, correlation with the fecundity of the queens, and ubiquitous presence in different queen tissues, suggest a role other than participation in chemical communication for this compound. A large spectrum of protective biological functions are ascribed to the two naturally occurring geometric isomers of nerolidol, including antimicrobial, antifungal and antioxidant properties (Chan et al. 2016) that may be beneficial for the long-lived and highly metabolically active physogastric queens.

In summary, we confirm the remarkable simplicity of the chemical alphabet of termite pheromone communication, using only a handful of different pheromone components secreted from an evolutionary-conserved set of exocrine glands. Their species-specific qualitative and/or quantitative combinations, together with other recognition and orientation cues ensure a wide range of functions from trail-following and recruitment to mate attraction (Bordereau and Pasteels 2011). Last but not least, the biosynthetic machinery producing the C_{12} alcohols has recently been observed to play additional roles in communication, being co-opted by the frontal glands of soldiers in an inquiline termite species and used for spatial segregation of the nest parasite colony from the colony of the nest builder (Jirošová et al. 2016).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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