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ORIGINAL ARTICLE

Ergatoid reproductives in the Neotropical termite *Nasutitermes aquilinus* (Holmgren) (Blattaria: Isoptera: Termitidae): developmental origin, fecundity, and genetics

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Abstract Termite colonies are usually headed by primary reproductives, which establish nests during the swarming season. However, secondary reproductives may develop in some species and become supplementary or replacement breeders, extending colony lifespan. Here we investigate the developmental origin, fecundity and genetic characterization of ergatoid reproductives in the Neotropical termite *Nasutitermes aquilinus* (Holmgren), using morphometrical and histological techniques, five microsatellite loci and the COI mitochondrial DNA. Twelve measurements performed on 208 apterous individuals of *N. aquilinus* revealed 10 groups, including ergatoid females, which developed from major workers through two successive molts, and were characterized by the presence of imaginal features such as eyes and wing buds. The differentiation of these features was correlated to physogastric development in these ergatoids. Histology revealed oocytes in all maturation stages in worker-derived reproductives of *N. aquilinus*, presence of nonflagellate spermatozoa inside the spermatheca, and royal fat body. Thus, ergatoid reproductives were reproductively functional. According to the genotypes of 221 individuals from 11 nests, and mitochondrial haplotypes of 43 ergatoids, 73% of the colonies were simple families, whereas 27% were extended families. Despite the occurrence of related reproductives, low inbreeding rates were detected within and among colonies. Such values could be explained given that sib mating itself cannot result in a higher inbreeding rate but depend on several factors discussed in detail. This is the first study to investigate the genetic structure of termite colonies influenced by the development of ergatoids, and further investigations are encouraged to understand the influence of these reproductives on colony lifespan.

Key words breeding system; caste differentiation; COI; genotyping; microsatellites; reproduction

Introduction

Termite colonies are usually headed by primary reproductives, termed king and queen, which establish the new colonies after swarming (Roisin, 2000). This event may be influenced by several factors and tend to be synchronized among colonies, increasing the likelihood of unrelated

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partners founding a new nest (Mitchell, 2008). In many species, the breeding tasks of the colonies are performed by secondary reproductives when the primary reproductives are absent. These individuals are neotenics when differentiated from immatures or adultoids when differentiated from winged sexuals (Myles, 1999). Neotenic reproductives develop from workers, referred to as ergatoids, or from nymphs, referred to as nymphoids (Roisin, 2000), and their occurrence is less prominent within the so-called higher termites, in which these individuals are replaced by adultoid reproductives in some Termitidae lineages (Myles, 1999).

Sexual reproduction is predominant within Isoptera, resulting in a higher genetic diversity which has a positive impact on colony survival during environmental stress. Some species conditionally alternate from sexual to asexual reproduction, solving this breeding tradeoff (Matsuura, 2011). Thus, while the genetic diversity is maintained through sexual reproduction, the queen may adopt asexual reproduction to duplicate her genetic contribution and increase within-colony relatedness (Matsuura *et al.*, 2009). In both sexual and asexual reproduction, the secondary reproductives may extend the colony lifespan (Vargo, 2019).

Termite colonies may be classified into the following breeding structures: simple families, in which a single pair of monogamous reproductives and its progeny are present; extended families, in which multiple related reproductives descend from a royal couple and perform the reproductive tasks; and mixed families, characterized by the coexistence of unrelated reproductives and their progeny (Vargo & Husseneder, 2011; Vargo, 2019). In any case, many factors are responsible for the genetic structure of the colonies, including number and relatedness among primary and secondary reproductives, and the number of descendant generations (Thorne *et al.*, 1999; Vargo & Husseneder, 2011).

The genus *Nasutitermes* is the most diverse in number of species within Isoptera and show plentiful variety of breeding mechanisms. The occurrence of multiple secondary reproductives is common, which exhibit an important role in egg-laying rate and contribute to the population growth (Roisin & Pasteels, 1986, 1987; Lefeuve, 1987; Noirot & Thorne, 1988). Few studies have focused on the genetic structure within *Nasutitermes*, shedding light on the origins and relatedness of the reproductives, as well as the inbreeding rate in colonies headed by them (Atkinson & Adams, 1997; Thompson & Hebert, 1998; Adams *et al.*, 2007). However, no genetic investigation has focused on the ergatoid reproductives, which differentiate in about 36% of the *Nasutitermes* spp. studied up until now (Silva *et al.*, 2018, unpublished data).

The Neotropical termite *Nasutitermes aquilinus* (Holmgren) is an arboreal nest-building species classified as an opportunistic pest in rural buildings (Torales, 1998; Fontes & Milano, 2002). Colonies of *N. aquilinus* are generally headed by a founding pair but, according to Torales and Coronel (2006), about 20% of the colonies develop ergatoid reproductives during their lifecycle. Ergatoid reproductives may be numerous when present, occurring in dozens or even hundreds of individuals, but only their external morphology has been examined in previous research studies (Fontes & Terra, 1981; Torales & Coronel, 2006). Thus, this study aimed to explore the developmental origin, fecundity and genetics of the ergatoid reproductives of *N. aquilinus*, trying to answer the following fundamental questions: How do ergatoid females differentiate within the apterous line? Are those individuals functional reproductives? How are the colonies of *N. aquilinus* genetically structured? To answer these questions, we used different methodological approaches, including morphometry, histology, and genetic analyses using microsatellite markers and mitochondrial DNA sequencing.

Materials and methods

Sampling of termites

A total of 12 nests (#1 – #12) of *N. aquilinus* (Holmgren, 1910) were collected from urban and agricultural areas in the cities of Rio Claro (22°24′43.9″S 47°36′34.0″W), São Carlos (21°59′26.7″S 47°52′58.3″W), Corumbataí (22°13′26.2″S 47°38′26.1″W), and Itapetininga (23°35′4.1″S; 48°1′59″W), State of São Paulo, Brazil (Fig. 1, Table 1).

Morphometry

Sampled termites from #1, #2, and #12 were fixed in FAA (formaldehyde, absolute alcohol, and glacial acetic acid, 1 : 3 : 1) during 24 h and transferred to 80% ethanol. All the samples were analyzed under stereomicroscope Zeiss Stemi SV11 (Zeiss, Jena, Germany) and 12 different measures were obtained following the methods described by Haifig and Costa-Leonardo (2016). In total, 208 apterous individuals were measured: 45 larvae, 30 minor workers, 30 major workers, 15 minor soldiers, 45 major soldiers, 30 presoldiers, 3 preneotenics, and 10 ergatoid females. The measures used were: (1) head length (HL), including the nasus in the case of soldiers; (2) maximum head width (HW1); (3) head width at the base of the mandibles (HW2); (4) maximum head height without postmentum (HH); (5) labrum width (LW); (6) pronotum

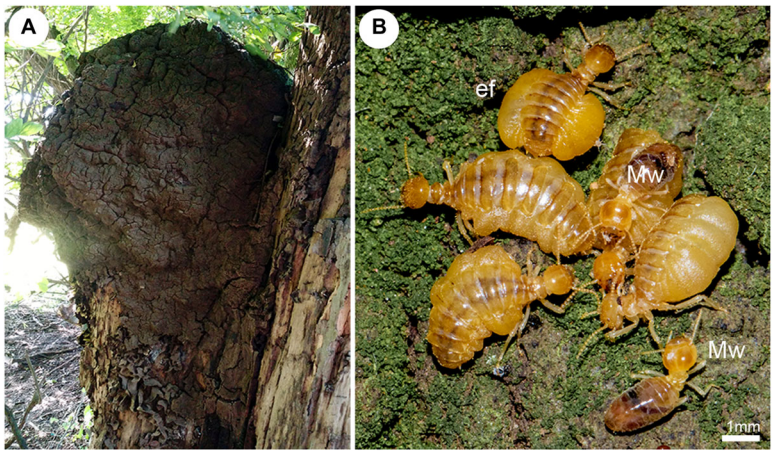

Fig. 1 (A) Arboreal nest of *Nasutitermes aquilinus*. (B) Ergatoid females (ef) and major workers (Mw).

length (PL); (7) pronotum width (PW); (8) mesonotum length (MsL); (9) metanotum length (MtL); (10) tibia length (TL); (11) femur length (FL); and (12) femur width (FW). Morphometric data were submitted to a principal component analysis (PCA). The scores of PC1 and PC2 were analyzed using ANOVA (one-way), followed by the Tukey's test ($P < 0.05$). In addition, to differentiate the degrees of cuticle sclerotization (tanning) among major workers, preneotenics, and ergatoid females, these individuals were photographed under a stereomicroscope (Zeiss Discovery V8, Zeiss, Jena, Germany) and the average values of HSB color model (hue angle, saturation, brightness) were calculated (Tsuchida *et al.*, 2010; Masuoka & Maekawa, 2016). The head of each individual was overlapped with 10 grids using the software Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA), and the HSB extracted from each grid using the color picker. Data were analyzed using the lme function in the nlme library to fit a mixed-effects model in R (version 3.6.0, R Core Team, 2019), considering HSB values and stage (major workers, preneotenics, and ergatoids) as fixed effects and the nest of origin and each individual as random effects. Data were log transformed to achieve both homoscedasticity and normality, and a model simplification procedure was performed to estimate the *P* values of the interaction terms (Crawley, 2007).

In order to evaluate the physogastry of ergatoid females, we followed the methodology described by Roisin and Pasteels (1986) and Costa-Leonardo *et al.* (1998). For this purpose, the abdominal length (distance between the first and seventh tergites) and the width of the fourth tergite were measured from 29 ergatoid females (25 from nest #6 and 4 from nest #11). All the measures were performed under stereomicroscope Zeiss Discovery V8 (Zeiss, Jena, Germany) and used to calculate the abdominal volume based on the cylinder shape. To estimate a correlation between physogastry and development of imaginal features, the right anterior wing bud length was also measured, and the data were subjected to the Pearson's correlation coefficient. All statistical tests were performed using R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Histology

To evaluate the development and maturation of the reproductive systems in ergatoid females, we sectioned the abdomens of females from nests #6 and #11 near the sixth tergite, and the posterior region was fixed in FAA during 24h. Then, the samples were dehydrated in a standard ethanol series (70%–95%), embedded in Historesin (Leica, Byosystems, Buffalo Grove, IL, USA) plus catalyzer for polymerization. Sections 3μm thick were obtained using a microtome Leica RM 2245, mounted on histological slides and stained with toluidine blue/fuchsin. The material was analyzed and documented under Leica ICC50 photomicroscope (Leica Microsystems, Wetzlar, Germany), using the software LAS v4.0 (Leica Microsystems, Wetzlar, Germany). The oocyte developmental stages were classified according to Grandi *et al.* (1988) based on yolk deposition and follicular cell features.

Genetic analysis

Microsatellite development The DNA was extracted from the heads of seven workers of *N. aquilinus* using a DNeasy Blood and Tissue kit (Qiagen®, Germantown, MD, USA). All the samples were pooled to obtain a suitable quantity of DNA for subsequent sequencing. For library preparation, we used an Illumina MiSeq Reagent

Table 1 Location of the nests and sampled individuals of *N. aquilinus*.

Nest	Location (city/state)	Genotyping	Morphometry	Histology
#1	Corumbataí/SP	Workers (9) and soldiers (8)	Larvae (20), workers (30), presoldiers (15), soldiers (30)	–
#2	Rio Claro/SP	Ergatoid females (14), workers (15), and soldiers (16)	Larvae (25), workers (30), presoldiers (15), soldiers (30), preergatoids (3)	–
#3	Rio Claro/SP	Workers (12), soldiers (13), and nymphs (10)	–	–
#4	Rio Claro/SP	Workers (7) and soldiers (4)	–	–
#5	São Carlos/SP	Workers (10), soldiers (8), and nymphs (8)	–	–
#6	São Carlos/SP	Ergatoid females (25), workers (8), soldiers (12), and nymphs (7)	Ergatoid females (25)†	Ergatoid females (4)
#7	São Carlos/SP	Workers (7) and soldiers (10)	–	–
#8	São Carlos/SP	Workers (5) and soldiers (6)	–	–
#9	São Carlos/SP	Workers (6) and soldiers (7)	–	–
#10	São Carlos/SP	Workers (5) and soldiers (9)	–	–
#11	Rio Claro/SP	Ergatoid females (4), workers (6), and soldiers (7)	Ergatoid females (4)†	Ergatoid females (2)
#12	Itapetininga/SP	–	Ergatoid females (10)	–

†Individuals used to evaluate the physogastry.

Kit V3 (300 × 300 paired-end reads), and the sequencing was performed using an Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA). The obtained sequences were *de novo* assembled using the program SparseAssembler (Ye *et al.*, 2012). The obtained DNA sequences (contigs) were screened using Msatcommander® software (Faircloth, 2008) to identify those with microsatellites. We identified 118 sequences containing microsatellites, from which 13 primer pairs were designed using the program Primer 3 v.4.0.0 (Untergasser *et al*., 2012). Forward primers were labeled with fluorescent dye FAM (Applied Biosystems, Foster City, CA, USA), optimized using PCR reactions and submitted to capillary electrophoresis on an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). For this purpose, workers from eight different nests were used, and tests revealed that only locus 2296 was polymorphic and suitable for further genetic analysis (Table 2).

Eight microsatellite loci developed for *Nasutitermes corniger* (Atkinson *et al.*, 2007), 12 for *Silvestritermes euamignathus* (Haifig *et al.*, 2016), and 9 for *Velocitermes heteropterus* (Lima *et al.*, 2013) were also cross-tested on samples of *N. aquilinus* (Table 2). Of the 29 additional loci tested, 4 proved suitable for use in *N. aquilinus*, giving 5 loci total used in this study.

Microsatellite genotyping A total of 92 workers, 103 soldiers, and 26 nymphs sampled from nests #1–#11, in addition to 43 ergatoid females from nests #2, #6 and #11, were genotyped (Table 1). The total DNA was individually extracted from the head and thorax of the samples using a Genomic DNA Purification Kit (Wizard®, Promega Corporation, Madison, WI, USA), following the manufacturer instructions. PCR reactions were performed in 12-μL volumes, containing 15 ng/μL of template DNA, ddH_2O, 5 mmol/L dNTPs, 5 U/μL *Taq* DNA Polymerase, 1.2 μL 1× PCR buffer, 0.5 mmol/L $MgCl_2$, 100 μmol/L of FAM-labeled forward primer, and 100 μmol/L of reverse primer. PCR products were run on an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Allele peaks were visualized using the software Geneious R10 (Biomatters Ltd, Auckland, New Zealand), using the microsatellite plugin. For each colony, the number of alleles was estimated using the Excel add-in Microsatellite toolkit, and *F*-statistics and relatedness values were calculated with the aid of FSTAT software (Goudet, 2002), using genotypes of workers, soldiers and nymphs.

Mitochondrial DNA sequencing The COI gene from 43 ergatoid females (#2, #6, and #11) was sequenced to identify the maternal lineages of the neotenics. PCR

Table 2 Microsatellite loci selected for genetic analysis.

Locus	Primer sequences	Repeat motif	Size (bp)	Source	GenBank accession number
Ncor3	F: GATCACTGTTG-GTTCAAGAGA R: ATGATACACC-CAAATGAAATG	$(TAA)_{26}$	61–88	Atkinson *et al.* (2007)	DQ514584
Na2296	F: ACACCCCTCTATC-CGCAAAA R: AGGTGAAA-GAACAGGCCCTT	$(ATT)_{20}$	66–162	Current study	MN216147
Vh19-1	F: ACTATTCCAGTG-GCTCCAA R: GGCTTCACATCTC-CCAAT	$(ATT)_{12}$	75–108	Lima *et al.* (2013)	MN216145
Vh22-1	F: GCAAACATGACCA-CAAGCAG R: GGTTCACCCTTCT-GTATCGAAT	$(ATT)_4$	127–136	Lima *et al.* (2013)	MN216146
Se5	F: ACTGAAC-GAGTTGTCTGCAA R: GGTTTCTTCCAT-GACCACCA	$(ATC)_8$	195–207	Haifig *et al.* (2016)	KU359003

reactions were carried out in 15 μL volumes, containing 15 ng/μL of template DNA, ddH_2O, 5 mmol/L dNTPs, 5 U/μL *Taq* DNA polymerase, 1× PCR buffer, 0.5 mmol/L $MgCl_2$, and 100 μmol/L each primer (forward ATGGCAGATTAGTGCAATGG and reverse GTTTAA-GAGACCAGTACTTG). PCR products were purified with ExoSAP-IT® (Thermo Fisher Scientific, Waltham, MA, USA), following the instructions of the manufacturer and submitted for Sanger sequencing, which was performed at Macrogen Inc, Seoul, South Korea. The sequences generated were 596 bp long, which were assembled and edited with Geneious R10 (Biomatters Ltd, Auckland, New Zealand).

Results

Morphometry

The PCA analysis separated all the apterous individuals of *N. aquilinus* into 10 groups, which were first and second larval instars, minor and major workers, minor and major presoldiers, minor and major soldiers, preneotenics and ergatoid neotenics. PC1 scores were responsible for 81.44% of the group split ($F_{9,198} = 485.5$; $P < 0.001$), and PC2 scores were responsible for 9.72% ($F_{9,198} = 359.8$; $P < 0.001$) (Fig. 2). All of the groups differed significantly with respect to the scores of PC1 or PC2, except major workers and preneotenics that did not differ in either PC1 ($P = 0.1536$) and PC2 ($P = 0.1486$) scores analyses.

The separation of the apterous castes of *N. aquilinus* into groups indicates the following developmental pathway (Fig. 3): larvae of first instar (L1) hatch from the egg and molt into larvae of second instar (L2). To this point, no sexual dimorphism is observed. The second molt leads male L2 to become minor workers or minor presoldiers, whereas females L2 become major workers. At this point, sexual dimorphism characteristic of the genus *Nasutitermes* becomes evident. On the one hand, in the male pathway, male L2 may differentiate into either a minor presoldier or a minor worker. Minor presoldiers become minor soldiers in a subsequent molt, and minor workers may molt either to another minor worker instar or to a major presoldier, which will become a major soldier with another molting event. On the other hand, in the female pathway, major workers can either molt to another major worker instar or to a preneotenic form, which will become a neotenic after a subsequent molt, the ergatoid.

Ergatoid females of *N. aquilinus* developed from major workers after two successive molts, including an

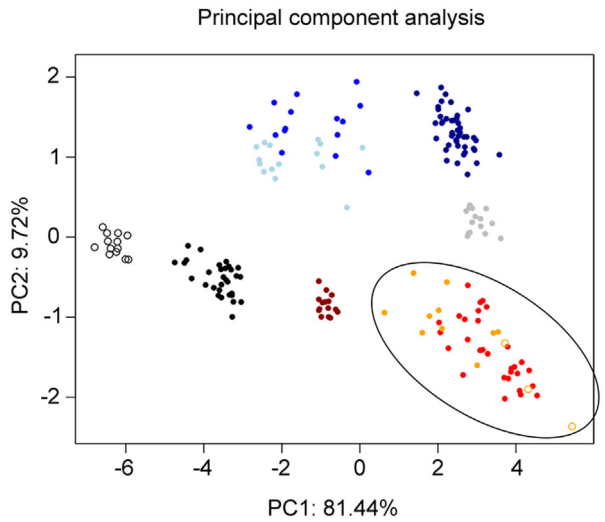

Fig. 2 Principal component analysis based on 12 measures performed on 208 apterous individuals of *Nasutitermes aquilinus*. Larvae 1 = white circles, larvae 2 = solid black, minor workers = solid dark red, major workers = solid red, preneotenics = orange circles, ergatoid females = solid orange, minor presoldiers = solid light blue, minor soldiers = solid blue, major presoldiers = solid gray, major soldiers = solid dark blue.

intermediate stage, the preneotenic form. Although major workers and preneotenics did not differ morphometrically, the latter were deprived of sclerotized cuticle and already exhibited some imaginal traits, such as compound eyes and wing buds (Fig. 4A, B). Ergatoid neotenics showed striking features when compared to the preneotenic form, including different degrees of cuticle sclerotization and conspicuous physogastry (Fig. 4C). The degrees of cuticle sclerotization, which may be inferred by the pigmentation of the cuticle after the protein tanning, significantly differed among major workers, preneotenics, and ergatoid females (mixed-effects model: $F = 960659.8$, df = 1,158; $P < 0.001$). The distribution of each group according to the HSB color model is given in Figure 4D. Ergatoid females from nest #6 showed a positive correlation between the length of the right anterior wing bud and physogastry ($r = 0.6528$, $t = 4.13$, df = 23, $P < 0.001$, Pearson's correlation coefficient). On the other hand, the results did not show correlation between physogastry and development of the wing buds in samples from nest #11, because three out of four ergatoids showed physogastry but no wing bud development.

Histology

Histological sections of the ergatoid abdomens showed ovarioles with oocytes in all developmental stages, including previtellogenic and vitellogenic stages (Fig. 5A–F). During the three previtellogenic stages, oocytes displayed variation in size and shape of the follicle cells, which showed a flat monolayer in stage I, cuboidal layer in stage II, and columnar layer in stage III (Fig. 5A–C). Yolk deposition started during the first vitellogenic stage (IV), also followed by the growth in size and changes in the follicular epithelium in the subsequent stages (V and VI). The terminal stage (VI) exhibited the maximum accumulation of yolk and the maximum size (Fig. 5F). In addition to oocyte maturation, some ergatoid females exhibited spermatheca with numerous nonflagellated and spherical spermatozoa, and royal fat body, a special tissue composed of columnar adipocytes with conspicuous nucleoli (Fig. 6A,B).

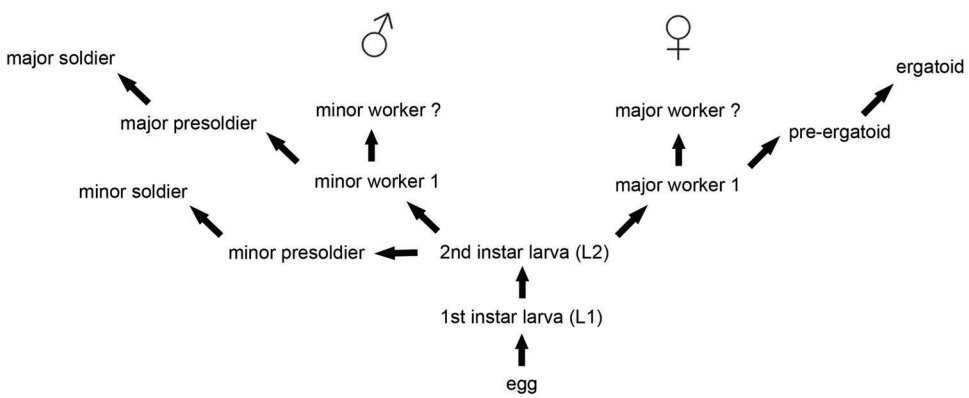

Fig. 3 Developmental pathway of the apterous line of *Nasutitermes aquilinus*. Male and female larvae 1 (L1) hatch from the egg and molt to larvae 2 (L2). Male L2 may molt to either a minor presoldier or a minor worker 1. Minor presoldiers molt to minor soldiers, whereas minor worker 1 molts to either another minor worker instar or major presoldiers. Female L2 molt to major worker, which may molt to either another major worker or to a preergatoid. This preneotenic stage molts to an ergatoid form.

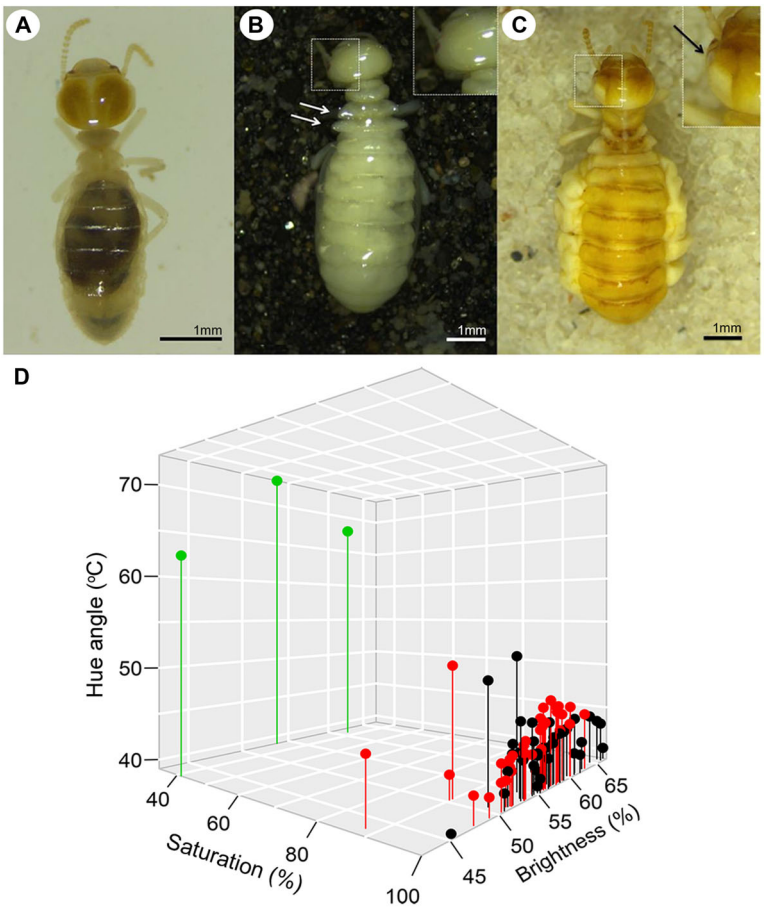

Fig. 4 Development of ergatoid females from major workers of *Nasutitermes aquilinus*. (A) Major worker. (B) Preneotenic form. (C) Ergatoid female. Note the development of imaginal traits in the preneotenic form and ergatoid, such as wing buds (white arrows) and compound eyes (magnification insert in B and C, and black arrow). (D) 3D-plot based on the HSB color model (hue angle, saturation, and brightness). Black plots indicate ergatoid females ($n = 36$), red plots indicate major workers ($n = 34$), and green plots indicate preneotenics ($n = 3$). According to the model, the degree of cuticle sclerotization significantly differed among the groups (mixed-effects model: $F = 960659.8$, df = 1,158; $P < 0.001$).

Genetic analysis

The five microsatellite loci presented an average of 3.5 alleles per locus, ranging from 2 to 6. The locus *Ncor3* was the most diverse, exhibiting six different alleles among the 11 colonies sampled, while gene diversity averaged 0.435 across loci (Table 3). Based on the number of genotypes at the five microsatellite loci, three colonies (#2, #3, and #6) were classified into extended families, because they exhibited genotype distributions inconsistent with monogamy, but did not share more than four alleles. Although physogastric ergatoids were collected from nest #11, the genetics of this colony indicated a simple family structure. In the remaining sampled nests, the number of alleles and genotypes also suggested the occurrence of simple families, headed by a single royal couple.

Mitochondrial sequencing data showed the occurrence of two different haplotypes among nests containing ergatoid reproductives. Colonies #2 and #6 shared the same haplotype, whereas colony #11 showed an exclusive COI sequence (Table 4). Only a single haplotype was observed within each colony, suggesting that all sampled ergatoid females were closely related, likely descending from a single matriline.

Overall, the sampled colonies experienced low degrees of inbreeding ($F_{it} = –0.312$), with moderate genetic diversity among them ($F_{st} = 0.166$). Within colonies, nestmates also exhibited notable genetic diversity ($F_{is} = –0.573$). The relatedness coefficient corroborated the occurrence of simple and extended families according to microsatellite genotyping and mitochondrial sequencing ($r = 0.482$).

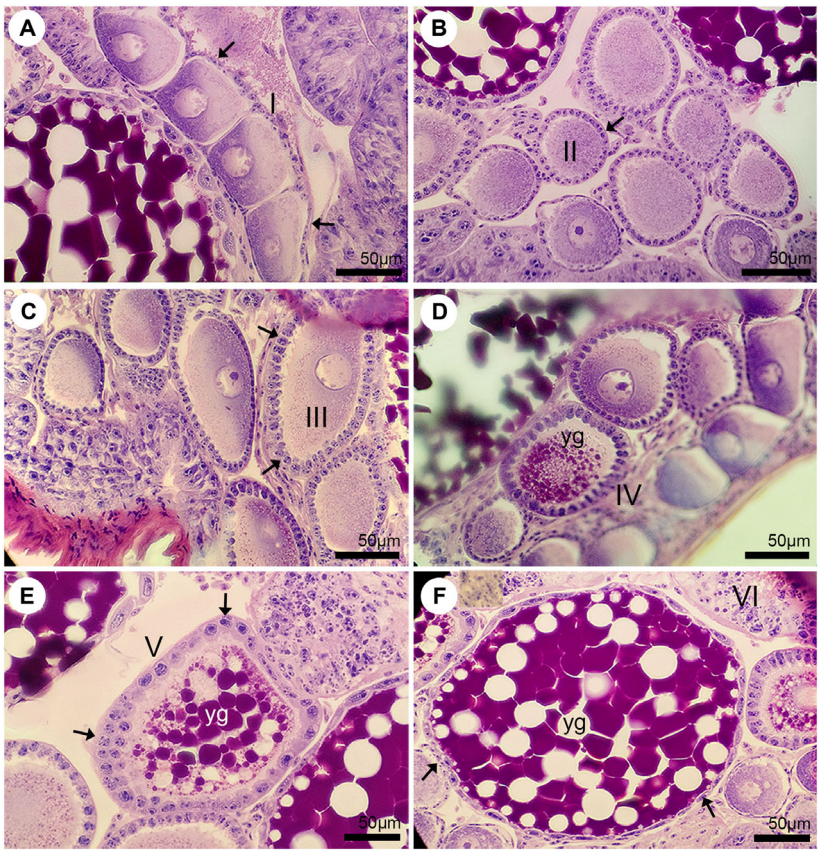

Fig. 5 Histological sections of the posterior abdomens of ergatoid females of *Nasutitermes aquilinus*, showing oocytes in different developmental stages. (A) First previtellogenic stage (I). Note the flattened follicular cells (arrows). (B) Second previtellogenic stage (II). Note the cuboidal epithelium of follicular cells (arrows). (C) Third previtellogenic stage (III). The oocyte grows in size and now possesses a follicle composed of columnar cells (arrows). (D) First vitellogenic stage (IV). The first yolk granules (yg) are deposited in the oocyte. (E) Second vitellogenic stage (V), characterized by the presence of a cuboidal epithelium (arrows) and yolk deposition. (F) Terminal vitellogenic oocyte (VI). Note the larger size, intense yolk deposition and flattened follicular cells (arrows). Stain: Toluidine blue/fuchsin.

Discussion

The differentiation of termite workers into ergatoid neotenics may occur after two or three successive molts (Roisin, 2000). According to our results, ergatoid females of *N. aquilinus* differentiated from major workers through two subsequent molts, including an intermediate form, the preneotenic stage, similar to the ergatoid developmental pathway described for the congeneric species *N. corniger* (Thorne & Noirot, 1982). The preergatoid stage of *N. aquilinus* is characterized by the presence of a pale cuticle, significantly clearer than major workers and ergatoids, and partial concealment of the gut due to the transformation of the fat body, similar to *N. novarumhebridarum* (Roisin & Pasteels, 1987).

All ergatoid neotenics studied were females and exhibited different degrees of physogastry. In fact, ergatoid males tend to be less frequently observed during nest surveys, probably due to their small size and mobility (Miura & Matsumoto, 1996; Torales & Coronel, 2006). Moreover, nests of *N. aquilinus* are often attached to tree trunks and the galleries penetrate the wood (Fontes & Terra, 1981; Cosarinsky, 2003), which result in sampling difficulties. Ergatoid reproductives of this species presented sclerotized cuticle and imaginal traits such as compound eyes and vestigial wing buds. Although the development of these features are attributed to the imaginal line, they are often recorded in worker-derived reproductives (Roisin & Korb, 2011), and within *Nasutitermes*, such records are even more frequent (Thorne & Noirot, 1982; Roisin &

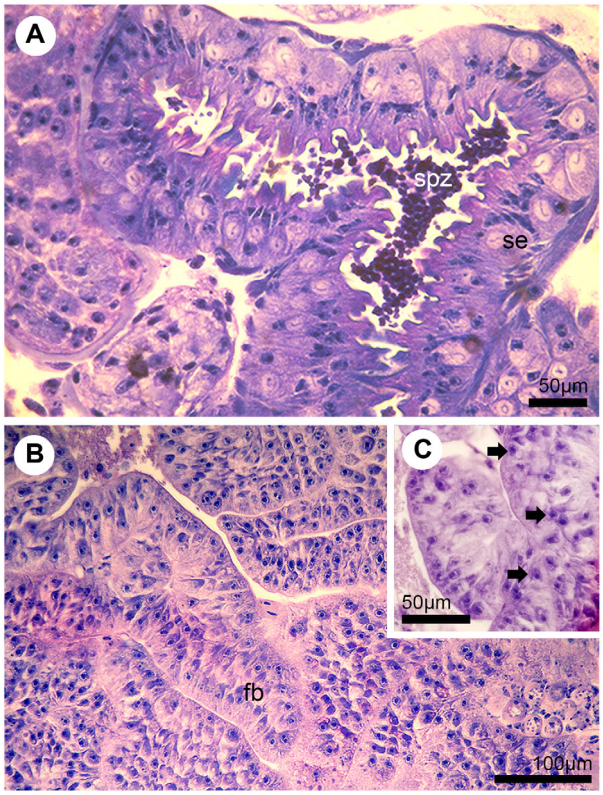

Fig. 6 (A) Transverse section of the posterior abdomen in an ergatoid female of *Nasutitermes aquilinus*. Several spherical and afflagelate spermatozoa (spz) are observed in the lumen of the spermatheca. (B) The royal fat body (fb), highlighting the columnar adipocytes. (C) Detail of the adipocytes. Note the nucleoli (arrows). se = spermatheca epithelium. Stain: Toluidine blue/fuchsin.

Pasteels, 1987; Noirot & Thorne, 1988; Miura & Matsumoto, 1996; Torales & Coronel, 2006). Probably, the genes responsible for the development of wing buds and compound eyes are linked to those related to the maturation of the reproductive organs, thus they may be expressed simultaneously during the differentiation of ergatoid reproductives (Noirot, 1985; Miura & Matsumoto, 1996; Miura, 2001).

Histological sections showed that ergatoid females of *N. aquilinus* were functional reproductives due to the presence of oocytes at different developmental stages, including terminal stages, associated with the royal fat body associated with expressive protein synthesis, a feature of functional queens in Termitidae. Thus, the royal fat body plays an important role in the metabolism of proteins involved in oocyte maturation (Han & Bordereau, 1982), and its association with the development of physogastry indicates a reproductive physiological

Table 3 Variability at five microsatellite markers in colonies of *N. aquilinus*.

Locus	Number of alleles	Gene diversity
Ncor3	6	0.478
Na2296	4	0.588
Vh19-1	3	0.462
Vh22-1	2	0.495
Se5	2	0.155
Mean ± SD	3.4 ± 1.67	
Overall		0.435

status of the individuals (Costa-Leonardo *et al.*, 2013). In addition, nonflagellated spermatozoa were observed in the spermatheca of the females, evidencing that these individuals mated inside the nest, even though no male reproductives were sampled. Spermatheca with spermatozoa was also observed in ergatoids of the congeneric species *N. guayanae* (Noirot & Thorne, 1988), and this is an indication of active reproduction.

The reproductive activity in some ergatoids may be limited because these individuals are less fecund when compared with other types of reproductives (Myles, 1999; Roisin & Korb, 2011). Many worker-derived reproductives of *N. corniger*, which differentiated after the death of the founding pair, could not contribute to the breeding tasks due to the occurrence of premature oocytes and degenerated ovaries (Thorne & Noirot, 1982). Additionally, Roisin and Pasteels (1987) reported that egg-laying started only 6 months after the differentiation of ergatoids in *N. novarumhebridarum*. Thus, in the evolution of termites, ergatoid forms seem to be less common than other reproductive types such as the nymphoids and adultoids (Roisin, 1990; Myles, 1999, 2000). However, since only ergatoid neotenics were sampled and because there is no record of nymphoid and/or adultoid reproductives in *N. aquilinus* (Myles, 1999), it is likely that only ergatoids differentiate and become functional reproductives in colonies of this species. Whether they differentiate in response to orphaning as replacement reproductives, or in the presence of the primaries as supplementary reproductives, is still unknown.

The diversity of alleles and genotypes within colonies of *N. aquilinus* suggested the occurrence of both simple (73%) and extended families (27%). None of the colonies showed more than four alleles at any locus or more than one mitochondrial haplotype, suggesting the lack of mixed families due to colony fusion or pleometrosis (Haifig *et al.*, 2016; Vargo, 2019). The reasons that lead a colony to adopt different breeding structures are variable, and include age of the colony, food availability and

Table 4 Haplotype variation for COI in *N. aquilinus* nests containing ergatoid reproductives.

Colony	GenBank accession number	Base pair position		
		151	502	632
#2 ($n = 14$)	MN240555	T	G	A
#6 ($n = 25$)	MN240556	T	G	A
#11 ($n = 4$)	MN240557	A	C	G

quality, colony-colony interactions, and environmental disturbance (Bulmer *et al.*, 2001; Aluko & Husseneder, 2007). Mixed families headed by neotenic reproductives have been reported for many termite groups, such as Mastotermitidae, Archotermopsidae, Kalotermitidae, Rhinotermitidae, and Termitidae, and are predominantly associated with nymphoids (Vargo & Husseneder, 2011; Vargo, 2019). Ergatoid differentiation in merging colonies seems to be rare in termites, with a single report for *Reticulitermes chinensis* (Huang *et al.*, 2013), and could sustain the absence of mixed families headed by ergatoids in *N. aquilinus*.

Despite the occurrence of four ergatoid females (some of which were inseminated) in #11, the genetic structure of the colony was consistent with monogamy. Thus, it is likely that these reproductives had not generated their first offspring yet, or it was composed of eggs and larvae, which were not genotyped in our analysis. Ergatoids from nest #11 shared two genotypes and a single COI haplotype, suggesting that they were siblings generated by a single reproductive pair and may have undergone recent differentiation. Similarly, neotenics from nest #6 were also offspring of a monogamous pair, whereas ergatoids from nest #2 showed more genotypes than those expected for offspring of a simple family colony. This observation on colony #2 allows us to raise two hypotheses: first, sampled ergatoid females were at least the second generation of neotenic reproductive, which developed within this colony, and may be the offspring of a preceding extended family. Second, #2 was a colony with pleometrotic foundation, and sampled ergatoids were result of reproductive cycles involving multiple related breeders.

According to Vargo and Husseneder (2011), sampled colonies of some termite species may present breeding structures of extended family, suggesting that the coexistence of sibling reproductives is essential during their lifecycle, as evidenced by the facultative parthenogenetic species *Silvestritermes minutus* (Fougeyrollas *et al.*, 2017). Given the occurrence of closely related reproductives, such colonies are likely to become more inbred ($F_{it} > 0$) (Dronnet *et al.*, 2005; Aldrich & Kambhampati, 2007; Husseneder *et al.*, 2008; Leniaud *et al.*, 2010). Despite the occurrence of extended families in *N. aquilinus*, including at least two possible generations of related reproductives in #2, a low inbreeding rate was observed within and among colonies. This low rate may be explained given that sib mating itself cannot result in higher inbreeding rates, but also depends directly on the number of extended families in the whole population, of functional breeders, and of inbred generations, as well as their reproductive rate (Thorne *et al.*, 1999; Vargo, 2019). For colony #6, whose ergatoids were offspring of a single parental pair, it is possible that this colony only recently developed neotenics so that it is still genetically similar to a simple family (Aldrich & Kambhampati, 2007; Huang *et al.*, 2013). This transition factor is also suitable for #11, given the observations discussed previously.

In conclusion, ergatoid females differentiate from major workers through a preneotenic stage, led by morphological and physiological changes (e.g., reproductive apparatus, eyes, wing buds). The presence of terminal oocytes and other reproductive features, such as spermatheca full of spermatozoa and the development of royal fat body, suggest that the sampled ergatoid females of *N. aquilinus* were reproductively functional. Although most of the studied colonies of *N. aquilinus* presented a simple family structure, indicating colonies headed by a monogamous pair, extended families were also evidenced by sampling of functional ergatoid reproductives and genetic analyses. The occurrence of ergatoid females with genotypes inconsistent with monogamous parents (colony #2) indicates two possibilities: (1) more than one generation of these reproductives differentiated within the colony and (2) such scenario was generated by multiple related primaries through pleometrosis during colony foundation or, less probably due to the nest architecture, as a result of colony fusion. This is the first study to investigate the genetic structure of colonies influenced by the development ergatoid reproductives, and further investigations are encouraged to provide more details on the long-term impact of ergatoid mating on colony development and longevity.

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Disclosure

The authors declare that they have no conflicts of interest regarding this article.

References

Adams, E.S., Atkinson, L. and Bulmer, M.S. (2007) Relatedness, recognition errors, and colony fusion in the termite *Nasutitermes corniger*. *Behavioral Ecology and Sociobiology*, 61, 1195–1201.

Aldrich, B.T. and Kambhampati, S. (2007) Population structure and colony composition of two *Zootermopsis nevadensis* subspecies. *Heredity*, 99, 443–451.

Aluko, G.A. and Husseneder, C. (2007) Colony dynamics of the Formosan subterranean termite in a frequently disturbed urban landscape. *Journal of Economic Entomology*, 100, 1037–1046.

Atkinson, L. and Adams, E.S. (1997) The origins and relatedness of multiple reproductives in colonies of the termite *Nasutitermes corniger*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 264, 1131–1136.

Atkinson, L., Adams, E.S. and Crozier, R.H. (2007) Microsatellite markers for the polygamous termite *Nasutitermes corniger* (Isoptera: Termitidae). *Molecular Ecology Notes*, 7, 299–301.

Bulmer, M.S., Adams, E.S. and Traniello, J.F.A. (2001) Variation in colony structure in the subterranean termite *Reticulitermes flavipes*. *Behavioral Ecology and Sociobiology*, 49, 236–243.

Cosarinsky, M. (2003) Comparative micromorphology of arboreal and terrestrial carton nests of the Neotropical termite *Nasutitermes aquilinus* (Isoptera: Termitidae). *Sociobiology*, 45, 1–15.

Costa-Leonardo, A.M., Soares, H.X. and Barsotti, R.C. (1998) Response to orphaning in two Neotropical termites: *Armitermes euamignathus* and *Embiratermes festivellus*. *Entomologia Experimentalis et Applicata*, 88, 109–114.

Costa-Leonardo, A.M., Laranjo, L.T., Janei, V. and Haifig, I. (2013) The fat body of termites: functions and stored materials. *Journal of Insect Physiology*, 59, 577–587.

Crawley, M.J. (2007) *The R Book*. Wiley, Chichester.

Dronnet, S., Chapuisat, M., Vargo, E.L. and Bagnères, A.G. (2005) Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. *Molecular Ecology*, 14, 1311–1320.

Faircloth, B.C. (2008) Msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources*, 8, 92–94.

Fontes, L.R. and Terra, P.S. (1981) A study on the taxonomy and biology the neotropical termite *Nasutitermes aquilinus* (Isoptera, Termitidae, Nasutitermitinae). *Revista Brasileira de Entomologia*, 25, 171–183.

Fontes, L.R. and Milano, S. (2002) Termites as an urban problem in South America. *Sociobiology*, 40, 103–151.

Fougeyrollas, R., Křivánek, J., Roy, V., Dolejšová, K., Frechault S, Roisin, Y. *et al*. (2017) Asexual queen succession mediates an accelerated colony life cycle in the termite *Silvestritermes minutus*. *Molecular Ecology*, 26, 3295–3308.

Goudet, J. (2002) FSTAT Software. Available in http://www2.unil.ch/popgen/softwares/fstat.htm. Access on 05 January 2015.

Grandi, G., Barbieri, R. and Colombo, G. (1988) Oogenesis in *Kalotermes flavicollis* (Fabr.) (Isoptera: Kalotermitidae). I. Differentiation and maturation of oocytes in female supplementary reproductives. *Bolletino di Zoologia*, 55, 103–122.

Haifig, I., Vargo, E.L., Labadie, P. and Costa-Leonardo, A.M. (2016) Unrelated secondary reproductives in the neotropical termite *Silvestritermes euamignathus* (Isoptera: Termitidae). *The Science of Nature*, 103, 9.

Haifig, I. and Costa-Leonardo, A.M. (2016) Caste differentiation pathways in the Neotropical termite *Silvestritermes euamignathus* (Isoptera: Termitidae). *Entomological Science*, 19, 174–179.

Han, S.H. and Bordereau, C. (1982) Ultrastrucuture of the fat body of the reproductive pair in higher termites. *Journal of Morphology*, 172, 313–322.

Huang, Q., Li, G., Husseneder, C. and Lei, C. (2013) Genetic analysis of population structure and reproductive mode of the termite *Reticulitermes chinensis* Snyder. *PLoS ONE*, 8, e69070.

Husseneder, C., Powell, J.E., Grace, J.K., Vargo, E.L. and Matsuura, K. (2008) Worker size in the Formosan subterranean termite in relation to colony breeding structure as inferred from molecular markers. *Environmental Entomology*, 37, 400–408.

Lefeuve, P. (1987) Replacement queens in the Neotropical termite *Nasutitermes coxipoensis*. *Insectes Sociaux*, 34, 10–19.

Leniaud, L., Dedeine, F., Pichon, A., Dupont, S. and Bagnères, A.G. (2010) Geographical distribution, genetic diversity and social organization of a new European termite, *Reticulitermes*

urbis (Isoptera: Rhinotermitidae). *Biological Invasions*, 12, 1389–1402.

Lima, J.T., Vargo, E.L. and Costa-Leonardo, A.M. (2013) Desenvolvimento de marcadores microssatélites para análises genéticas em *Velocitermes heteropterus* (Isoptera: Termitidae). Resumo do I Workshop sobre Insetos Sociais, Ribeirão Preto, SP.

Masuoka, Y. and Maekawa, K. (2016) Ecdysone signaling regulates soldier-specific cuticular pigmentation in the termite *Zootermopsis nevadensis*. *FEBS Letters*, 590, 1694–1703.

Matsuura, K. (2011) Sexual and asexual reproduction in termites. *Biology of Termites: A Modern Synthesis* (eds. D.E. Bignell, Y. Roisin & N. Lo), pp. 255–277. Springer, Netherlands, Dordrecht.

Matsuura, K., Vargo, E.L., Kawatsu, K., Labadie, P.E., Nakano, H., Yashiro, T. *et al*. (2009) Queen succession through assexual reproduction in termites. *Science*, 323, 1687.

Mitchell, J.D. (2008) Swarming flights of the fungus-growing termite, *Macrotermes natalensis* (Haviland) (Isoptera: Macrotermitinae), and the environmental factors affecting their timing and duration. *African Entomology*, 16, 143–152.

Miura, T. (2001) Morphogenesis and gene expression in the soldier-caste differentiation of termites. *Insectes Sociaux*, 48, 216–223.

Miura, T. and Matsumoto, T. (1996) Ergatoid reproductives in *Nasutitermes takasagoensis* (Isoptera: Termitidae). *Sociobiology*, 27, 223–238.

Myles, T.G. (1999) Review of secondary reproduction in termites (Insecta: Isoptera) with comments on its role in termite ecology and social evolution. *Sociobiology*, 9, 163–319.

Noirot, C. (1985) Differentiation of reproductives in higher termites. *Caste Differentiation in Social Insects* (eds. J.A.L. Watson, B.M. Okot-Kotber & C. Noirot), pp. 177–186. Pergamon Press, Oxford.

Noirot, C. and Thorne, B.L. (1988) Ergatoid reproductives in *Nasutitermes columbicus* (Isoptera, Termitidae). *Journal of Morphology*, 195, 83–93.

R Core Team (2019) A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Available in http://www.R-project.org/. Access: 26 April 2019.

Roisin, Y. (1990) Queen replacement in the termite *Microcerotermes papuanus*. *Entomologia Experimentalis et Applicata*, 56, 83–90.

Roisin, Y. (2000) Diversity and evolution of caste patterns. *Termites: Evolution, Sociality, Symbioses, Ecology* (eds. T. Abe, D.E. Bignell & M. Higashi), pp. 95–119. Kluwer Academic, London.

Roisin, Y. and Korb, J. (2011) Social organisation and the status of workers in termites. *Biology of Termites: A Modern Synthesis* (eds. D.E. Bignell, Y. Roisin & N. Lo), pp. 133–164. Springer: Netherlands, Dordrecht.

Roisin, Y. and Pasteels, J.M. (1986) Replacement of reproductives in *Nasutitermes princeps* (Desneux) (Isoptera: Termitidae). *Behavioral Ecology and Sociobiology*, 18, 437–442.

Roisin, Y. and Pasteels, J.M. (1987) Caste developmental potentialities in the termite *Nasutitermes novarumhebridarum*. *Entomologia Experimentalis et Applicata*, 44, 277–287.

Thompson, G.J. and Hebert, P.D.N. (1998) Population genetic structure of the Neotropical termite *Nasutitermes nigriceps* (Isoptera: Termitidae). *Heredity*, 80, 48–55.

Thorne, B.L., Traniello, J.F.A., Adams, E.S. and Bulmer, M. (1999) Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. *Ethology Ecology & Evolution*, 11, 149–169.

Thorne, B.L. and Noirot, C. (1982) Ergatoid reproductives in *Nasutitermes corniger* (Motschulsky) (Isoptera: Termitidae). *International Journal of Insect Morphology and Embryology*, 11, 213–226.

Torales, G.J. (1998) Rol de los isopteros en la Argentina. *Cupins: o desafio do conhecimento* (eds. L.R. Fontes & E. Berti Filho), pp. 413–135. Fundação Escola de Agricultura Luiz de Queiroz, Piracicaba.

Torales, G.J. and Coronel, J.M. (2006) Reproductive ergatoids in nests of *Nasutitermes aquilinus* (Isoptera, Termitidae, Nasutitermitinae). *Sociobiology*, 48, 209–221.

Tsuchida, T., Koga, R., Horikawa, M., Tsunoda, T., Maoka, T., Matsumoto, S. *et al*. (2010) Symbiotic bacterium modifies aphid body color. *Science*, 330, 1102–1104.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G. (2012) Primer3 - new capabilities and interfaces. *Nucleic Acids Research*, 40, e115.

Vargo, E.L. (2019) Diversity of termite breeding systems. *Insects*, 10, 52.

Vargo, E.L. and Husseneder, C. (2011) Genetic structure of termite colonies and populations. *Biology of Termites: A Modern Synthesis* (eds. D.E. Bignell, Y. Roisin & N. Lo), pp. 321–347. Springer, Netherlands, Dordrecht.

Ye, C., Ma, Z.S., Cannon, C.H., Pop, M. and Yu, D.W. (2012) Exploiting sparseness in de novo genome assembly. *BMC Bioinformatics*, 13, S1.

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