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BEHAVIORAL ECOLOGY – ORIGINAL RESEARCH

# Nest composition, stable isotope ratios and microbiota unravel the feeding behaviour of an inquiline termite

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## Abstract

Termites are eusocial insects having evolved several feeding, nesting and reproductive strategies. Among them, inquiline termites live in a nest built by other termite species: some of them do not forage outside the nest, but feed on food stored by the host or on the nest material itself. In this study, we characterized some dimensions of the ecological niche of *Cavitermes tuberosus* (Termitidae: Termitinae), a broad-spectrum inquiline termite with a large neotropical distribution, to explain its ecological success. We used an integrative framework combining ecological measures (physico-chemical parameters, stable isotopic ratios of N and C) and Illumina MiSeq sequencing of 16S rRNA gene to identify bacterial communities and to analyse termites as well as the material from nests constructed by different termite hosts (the builders). Our results show that (1) nests inhabited by *C. tuberosus* display a different physico-chemical composition when compared to nests inhabited by its builder alone; (2) stable isotopic ratios suggest that *C. tuberosus* feeds on already processed, more humified, nest organic matter; and (3) the gut microbiomes cluster by termite species, with the one of *C. tuberosus* being much more diverse and highly similar to the one of its main host, *Labiotermes labralis*. These results support the hypothesis that *C. tuberosus* is a generalist nest feeder adapted to colonize nests built by various builders, and explain its ecological success.

**Keywords** Isoptera · Termitidae · *Cavitermes tuberosus* · Humivorous · Neotropical · Nitrogen · Nest

## Introduction

Termites are eusocial insects having evolved several feeding and nesting strategies. They feed on microepiphytes, grass, dead or living wood, and humus (Eggleton and Tayasu 2001). Based on the gut content and the anatomy of workers, termites can be classified into four feeding groups reflecting the degree of decomposition of organic matter on which they feed (Donovan et al. 2001): group I and II are composed of wood, litter and grass feeders; termites of group III feed on very decayed wood or soil with high organic content; and group IV comprises feeders of soil with low organic content. While group I only contains lower termites, the other three groups consist of higher termites (Termitidae).

Another classification, based on lifetypes, describes the degree of association between nest site and food source (Abe 1987): single-piece nesters, which feed and nest in the same substrate; intermediate nesters, feeding both on their nest and outside; and separate-piece nesters, which actively forage outside. While the first two categories consist of only wood-feeding termites, termites of the third category display

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a broader range of possible diet. A fourth lifetype can also be recognized and consists of inquiline termites, living in nests built by another species (Shellman-Reeve 1997): their association ranges from facultative to obligatory inquilinism. Among obligatory inquilines, the best known examples are *Serritermes serrifer* (Serritermitidae) nesting only in nests of *Cornitermes* species (Termitidae: Syntermitinae) (Araujo 1977), and the genus *Inquilinitermes* (Termitidae: Termitinae) always nesting in the lower part of nests of *Constrictotermes* species (Termitidae: Nasutitermitinae) (Noirot 1970). The benefits of using a nest built by another species are numerous: inquilines avoid the cost of building and maintaining the nest, and profit from a shelter against environmental conditions and predators (Shellman-Reeve 1997). One major peculiarity in the lifestyle of some inquiline termites is that they do not forage outside the nest, but feed on food stored by the host or on the nest material itself, which may be considered a special form of humivory (Myles 1999; Bourguignon et al. 2011; Florencio et al. 2013). Most of the obligatory inquiline termites belong to the subfamily Termitinae from the Termitidae (Shellman-Reeve 1997).

The family Termitidae is the most phylogenetically recent (ca. 54 Mya; Bourguignon et al. 2015b, 2017) and species rich of all families (70% of described termites; Krishna et al. 2013a). It is characterized by the loss of cellulolytic flagellates and by displaying an entirely prokaryotic gut microbiota that brought along dietary diversification and tremendous ecological success (Bignell and Eggleton 2000; Eggleton and Tayasu 2001; Ohkuma and Brune 2011; Brune and Dietrich 2015). While it has long been thought that diet, gut microenvironment and vertical transmission were the primary determinants of bacterial communities (Abdul Rahman et al. 2015; Mikaelyan et al. 2015, 2017; Su et al. 2017), a recent study evidenced rampant horizontal transfer over evolutionary time between termite species with both vertical (colony-to-offspring) and horizontal (colony-to-colony) transmission (Bourguignon et al. 2018). Such mechanisms may be particularly important in inquiline–host pairs, but are yet to be investigated (Mikaelyan et al. 2015, 2017).

*Cavitermes tuberosus* (Emerson 1925) is a broad-spectrum inquiline colonizing arboreal nests built by other species, with a large neotropical distribution (Mathews 1977; Constantino 1991; Martius 1997; Apolinário and Martius 2004; Krishna et al. 2013b). This species was demonstrated to undergo asexual queen succession: queens sexually produce several generations of winged reproductives that disperse and colonize new nests, while they parthenogenetically produce numerous secondary queens that reproduce within their mother's nest, thereby increasing the colony reproductive output (Fournier et al. 2016; Hellemans et al. 2017b, 2019a). Also, these secondary reproductives are small, which may present the advantage of remaining able to circulate through the small galleries at the margins of the host's nest—a probable constraint for physogastric queens. These features appear particularly advantageous for species with an inquiline lifestyle. According to the classification of Donovan et al. (2001), *C. tuberosus* belongs to soil feeders of group III (Davies 2002; Davies et al. 2003). As it has never been observed to forage outside, this suggests that its main or exclusive food source may be the nest material of other termite species. To test this hypothesis, we used an integrative framework combining classical ecological measures with next-generation sequencing. More specifically, we (1) investigated for signs of nest consumption by analysing 26 physico-chemical parameters of nests corresponding to several stages of colonization by *C. tuberosus*; (2) measured the ratios of stable isotopes of nitrogen and carbon in nests and termite tissues to determine whether *C. tuberosus* feeds on nest material, i.e., on already processed, more humified organic matter (Hood-Nowotny and Knols 2007; Hyodo et al. 2008; Potapov et al. 2019); (3) determined the composition of bacterial communities present in the nests and guts of *C. tuberosus* using Illumina high-throughput sequencing of the bacterial 16S rRNA gene, to see if *C. tuberosus* shares gut microbiota with its hosts, irrespective of termite phylogeny (Mikaelyan et al. 2015; Bourguignon et al. 2018); and (4) identified OTUs potentially involved in the process of nitrogen fixation, a process that would balance the loss of nitrogen through the remineralisation of the nest material, which may be of importance in arboreal systems.

## Materials and methods

### Study site and model organism

The study was conducted in the tropical rainforest surrounding the Petit Saut dam area (N 05.07°, W 52.87°) in French Guiana. The mean annual temperature is 26 °C, and this area experiences approximately 3000 mm of rainfall with peaks in December–January and April–June. The region is covered by mature lowland forest with canopy height reaching 30–40 m on a well-drained lateritic tropical soil (de Granville 1988; Cosson et al. 1999).

According to our extensive sampling in French Guiana between 2012 and 2017, *C. tuberosus* lives in arboreal structures: (1) in an indistinct layer of soil material covering the roots or on the basis of tree trunks, in cavities within tree trunks, between the stems of palm trees (*Astrocaryum* spp., Arecaceae), (2) in mounds against trees or well-defined arboreal nests built by other termite species; in this case, the nest may be abandoned or still inhabited by the initial builder, and sometimes by other colonizing species. If *C. tuberosus* shares a nest with another species, they live in separate galleries. *Cavitermes tuberosus* was mostly found in arboreal nests of *Labiotermes labralis* and *Silvestritermes*

*heyeri*, rarely in nests of *Silvestritermes minutus* (all belonging to the Termitidae: Syntermitinae), in the uppermost part of mounds build by the *Termes fatalis* species complex (sensu Hellemans et al. 2017a) and in nests of *Neocapritermes taracua* (Termitidae: Termitinae) at the base of trees. Co-occurring species with *C. tuberosus* in abandoned nests were *Nasutitermes* spp. and *Subulitermes*-group species (Termitidae: Nasutitermitinae), and *Anoplotermes*-group species (Termitidae: Apicotermitinae).

## Ecological measurements

### Physico-chemical analyses of termite nests

In 2017, we collected 39 termite nest fragments in the Petit Saut dam area, French Guiana (Table S1). Approximately 50 g of nest soil material was obtained by gently crushing a portion of nest with a pestle and mortar to obtain fine aggregates. Soil samples were dried in an oven at 60 °C until measurements were made. Nest fragments were divided into two categories reflecting the colonization stage by *C. tuberosus*: category A included nests inhabited by their builder only (*L. labralis*, *N. taracua*, *S. heyeri*, or *T. fatalis*; $n = 12$) and category B included nests in which *C. tuberosus* coexisted with the nest builder ($n = 5$), or nests with *C. tuberosus* only ($n = 22$).

We used 26 physico-chemical parameters to characterize nest material samples. First, we measured four classical parameters according to standard protocols (Pansu and Gautheyrou 2006): $pH_{H2O}$, electrical conductivity (EC, in mS $cm^{-1}$), organic matter content (OM) and the relative proportion of humic and fulvic acids in the OM. Nest pH and EC were measured on a 1:5 sample:deionized water (v/v) blend after 2 h agitation, respectively, with glass electrodes (Mettler-Toledo) and a conductimeter (VWR EC300). OM in the nest was estimated by sample mass loss after dry ashing for 12 h at 500 °C. Fulvic acids are comparatively more abundant than humic acids in topsoils while they equilibrate in the nests, as well as in faeces, after gut passage (Garnier-Sillam and Harry 1995; Brauman 2000; Ji et al. 2000). The humification stage was quantified using the extinction coefficient in visible light (E4/E6) which reflects the relative proportion of humic and fulvic acids in the OM. Humic acids are characterized by ratios below 5.0, while ratios of fulvic acids are between 6.0 and 8.5, so the lower the ratio, the higher the proportion of humic relative to fulvic acids in the solution (Schnitzer 1971). This coefficient was obtained by measuring the absorbance on a UV–VIS Hitachi U-2900 spectrophotometer (Tokyo, Japan) at 465 and 665 nm of a suspended solution of nest soil incubated under agitation with 0.05 M $NaHCO_3$ at pH 8.5 for 24 h and centrifuged for 5 min at 5000 rpm.

Second, we quantified the concentration of 18 bioavailable—essential (B, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, P, S, Zn), non-essential (Al, Ba) and toxic (Cd, Pb)—elements in the nest (dry weights, expressed in µg $g^{-1}$) after extraction with 0.5 M $NH_4$-acetate 0.03 M EDTA at pH 4.65 during 1 h and subsequently quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES) with CCD detector (Varian, Vista MPX). Finally, we determined the total C and N contents (%) and isotopic ratios (hereafter $\delta^{13}C$ and $\delta^{15}N$, respectively) from nest samples (see below). Raw data of the 26 physico-chemical parameters of nests are given in Supplementary File 1.

### Isotopic analyses

Termite tissues from all species ($n = 42$ colonies, from five species), nests ($n = 39$; Table S1) and samples from the topsoil layer ($n = 4$) were subjected to C and N isotopic analyses. To avoid contamination from gut content, we dissected heads of 25 workers and directly stored them in 96% ethanol at − 20 °C until use. Storage in 96% ethanol preserves tissues for morphological and genetic analyses and isotopic ratios are not altered (Florencio et al. 2011). Tissue samples were dried at 60 °C for 24 h in a stove prior to weighing. Because carbonate isotopic composition is generally different from organic carbon isotopic composition, carbonates must consequently be removed from samples if present: their absence from dried samples of nests and topsoil was ascertained by reaction with HCl 10%, and no acidification was therefore performed. Replicates of 0.50–1.00 mg and 5–10 mg of dried termite tissues and nest samples, respectively, were weighted in tin capsules using a Mettler AT261 DeltaRange (Mettler-Toledo) precision balance (0.01 mg).

All samples were analysed for $\delta^{13}C$ and $\delta^{15}N$ (‰) via continuous flow elemental analysis–isotope ratio mass spectrometry (CF–EA–IRMS) at the University of Liège (Belgium) using a vario MICRO cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) coupled to an IsoPrime100 mass spectrometer (Isoprime, Cheadle, United Kingdom). Sucrose (IAEA-C6; mean ± SD: $\delta^{13}C = -10.8 \pm 0.5$‰) and ammonium sulphate (IAEA-$N_2$; $\delta^{15}N = 20.3 \pm 0.2$‰) were used as certified reference materials (CRM). Both CRMs are calibrated against international isotopic references, i.e., the Vienna Pee Dee Belemnite (VPBD) for carbon and atmospheric air for nitrogen. The standard deviations of the multi-batch replicate measurements of lab standards (amphipods) as well as glycine (Merck, Darmstadt, Germany) interspersed among the samples were 0.1‰ and 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. Raw data are given in Supplementary File 1.

### Statistical analyses

All following analyses were performed using R *v*3.1.3 (R Development Core 2015). To investigate for changes in the 26 measured physico-chemical parameters of nests colonized by *C. tuberosus*, we first performed a principal component analysis (PCA; for details, see Supplementary Figure S1) to describe the overall changes between nest categories and plotted the first two principal components (PC) using the ggord package (Beck 2017). Additionally, we performed a linear discriminant analysis (LDA; "lda" function from the MASS package). Prior to LDA, we performed log transformation and standardization of variables, excluding collinear variables (at threshold 0.70; "vifcor" function from the usdm package) and verifying multivariate homogeneity of within-group covariance matrices ("betadisper" function from the vegan package). Significance of pre-defined categories was determined using a Welch two-sample *t* test on the scores of the first linear discriminant (LD1). Finally, we performed Wilcoxon rank-sum tests on each original variable and corrected *p* values for multiple comparisons using the false discovery rate (FDR) method (Benjamini and Hochberg 1995).

Isotopic niches of each species were inferred from the biplot of $\delta^{13}$C and $\delta^{15}$N and were computed as Bayesian standard ellipses using SIBER (SIAR package) (Parnell et al. 2010; Jackson et al. 2011). Diet spaces were compared using MANOVA on both $\delta^{13}$C and $\delta^{15}$N followed by univariate analyses using Kruskal–Wallis rank-sum tests and pairwise species comparisons with post hoc Nemenyi's tests (from PMCMR package).

### Diversity and composition of bacterial communities from termite guts and nests

Wild mature workers of *C. tuberosus* and several of its hosts (*L. labralis*, *N. taracua*, *S. heyeri*, *S. minutus* and *T. fatalis*) were collected from 23 nests in March 2016 and January 2017 (for details see Table S1). Individuals were cold immobilized and surface cleaned with 80% ethanol and 1X PBS. Whole guts (WG) from 26 samples (Table S1; each in triplicate; $n \approx 30$ WG per replicate) were dissected with sterile forceps, pooled and directly preserved on site in liquid nitrogen (in few cases more than one species inhabited a single nest). Additionally, triplicates of approximately 250 mg were preserved from 19 nest samples (Table S1). Nucleic acids from both whole guts and nest samples were extracted using Power Viral Environmental RNA/DNA Isolation Kit (MO-BIO) with 0.1 mm glass beads, following manufacturer's instructions supplemented by a cell lysis step by bead-beating at 20 Hz for 2 min. In the case of nest samples, the extraction was preceded by crushing the piece of mound with sterile mortar and by shaking the resulting powder in a tube with glass beads and 750 µL of buffer PV1 for 45 min at 500 rpm at 22 °C. The obtained DNA was treated with 1 µL of 10 µg/mL RNase A (Sigma) at room temperature (RT) for 30 min and quantified by Qubit dsDNA HS Assay Kit. The bacterial 16S rRNA gene libraries were prepared using an Illumina platform-compatible approach as previously applied by Marynowska et al. (2017) in the study of the termite gut bacterial communities.

After demultiplexing and quality trimming, resulting sequencing reads were assigned to operational taxonomic units (OTUs) at 97% similarity with *Usearch* pipeline *v*7.0.1090_win64 (Edgar 2010), and further taxonomically annotated with SILVA database *v*128 (Pruesse et al. 2007) using *mothur* software *v*1.38.0 (Schloss et al. 2009). Due to high reproducibility of triplicates (Fig. S2; Table S3), reads resulting from replicates were pooled and analysed together. Additionally, to ensure even coverage across samples, sequences were subsampled to 10,000 reads per sample. The average pairwise Bray–Curtis distance for each gut bacterial community of *Cavitermes* and their hosts were calculated using *mothur* ("dist.shared" function), visualized using principal coordinate analysis (PCoA; "pcoa" function in *mothur*), and compared statistically using Wilcoxon signed-rank test (different if $p < 0.001$). Statistical analyses were performed using *mothur* and R environment (R Development Core 2015) on the OTUs annotated as of prokaryotic origin. The quality-checked dataset is available in the GenBank repository under project accession KBWO01000000 (see details in Supplementary Table S2).

In addition, we investigated for the presence of bacteria potentially involved in nitrogen fixation, which may be of importance in arboreal systems. To do so, we blasted our 16S rRNA sequences against the complete prokaryotic RefSeq genomes database from NCBI. Using the threshold of 97.0% identity, OTUs potentially involved in the process of nitrogen fixation in gut as well as nests samples were identified, based on the presence of genes encoding for nitrogenase (*nif* subunits) in the matched genomes.

## Results

### Ecological measurements on nests colonized by *C. tuberosus*

PCA revealed a strong overlap between the two nest categories (for details, see Supplementary Fig. S1). Prior to LDA, 5 of the 26 log-transformed and standardized parameters of nests were excluded due to collinearity (Fe, Cd, Cr, Co and C): the first 4 strongly covaried among them and with Pb, as well as Fe and Co with Al, Cr with Cu and Ni, while C covaried with OM and N. The first linear discriminant (LD1) correlated the most with Al and Cu (13.42% and 11.90%, respectively), and nest categories significantly differed

on LD1 (Welch two-sample $t$ test: $t = -10.88$, $df = 17.76$, $p < 0.001$).

Wilcoxon rank-sum tests on the 26 physico-chemical parameters of nests showed that OM ($p = 0.049$) and $\delta^{13}C$ values ($p = 0.012$) decreased in nests colonized by *C. tuberosus* (category B) compared with those inhabited by the builder only (category A; Table S6), while the concentration of Al, Cd, Co, Cr, Cu, Fe, Mg, Ni and Pb (all $p < 0.05$) was higher in B nests. When corrected for multiple comparisons with the FDR method, only the effects of $\delta^{13}C$, Al, Cd, Co, Cr, Cu and Fe remained significant (all $p < 0.05$). Also, humification stage of nests could not be differentiated and similar proportions of humic and fulvic acids were found in A and B nests (Table S6; Welch two-sample $t$ test: $t = -0.75$, df = 15.81, $p = 0.46$), and the C:N ratio of nests was similar between A and B nests (Wilcoxon rank-sum tests; $W = 211$, $p = 0.299$).

## Isotopic analyses on nest material and termite tissues

Isotopic $\delta^{13}C$ values ranged from –26.4 to –30.1‰ and $\delta^{15}N$ values from 1.8 to 6‰ for nest samples ($n = 37$), and were similar to those from topsoil samples ($n = 4$; Fig. 1a). The C:N ratio of nests was positively correlated to $\delta^{13}C$ (linear regressions; $R^2 = 0.30$, $F_{1,35} = 15.09$, $p < 0.001$), and negatively to $\delta^{15}N$ ($R^2 = 0.22$, $F_{1,37} = 10.35$, $p = 0.003$). Nests inhabited by *C. tuberosus* exhibited a significant enrichment in $^{13}C$ compared to A nests (Table S6; Wilcoxon rank-sum tests; $W = 68$, $p = 0.012$), while $\delta^{15}N$ values marginally increased ($W = 100$, $p = 0.06$).

In termite tissues, isotopic ratios ranged from –22.7 to –28.3‰ for $\delta^{13}C$ and from 5.9 to 14.1‰ for $\delta^{15}N$ ($n = 138$). Overall, species differed in their stable isotopic ratios on both dimensions (Fig. 1b; MANOVA; Wilks' $\lambda = 0.14$, $F_{4,133} = 54.48$, $p < 0.001$), and follow-up univariate analyses indicated that species differed in both $\delta^{15}N$ and $\delta^{13}C$ values (Kruskal–Wallis tests; $\delta^{15}N$: $H_4 = 63.52$, $p < 0.001$; $\delta^{13}C$: $H_4 = 95.57$, $p < 0.001$). Most species differed in $\delta^{15}N$ values (post hoc Nemenyi's test; all $p < 0.04$) except for comparisons of *C. tuberosus* to *L. labralis* and to *S. heyeri*; *N. taracua* to *S. heyeri* and to *T. fatalis*. For $\delta^{13}C$ values, differences were observed between *C. tuberosus* and all species, and between *L. labralis* and *T. fatalis* (post hoc Nemenyi's test; all $p < 0.02$). Also, isotopic niches of species never overlapped in nests in which both the builder and *C. tuberosus* cohabited (Fig. S3).

*Cavitermes tuberosus* exhibited the broadest range of values for both isotope ratios ($\delta^{13}C$ range: 3.5‰; $\delta^{15}N$ range: 6.8‰; Table S7), and values were positively correlated with those of their nests (linear regressions; $\delta^{13}C$: $R^2 = 0.77$, $F_{1,75} = 252.6$, $p < 0.001$; $\delta^{15}N$: $R^2 = 0.38$, $F_{1,78} = 48.16$, $p < 0.001$). Finally, the isotopic niche of *C. tuberosus* did not differ when considering the nest builder identity (Fig. S4).

## Diversity and composition of bacterial communities from termite guts and nests

The diversity and composition of the gut and the nest microbiota of *C. tuberosus* and its hosts were assessed by high-throughput 16S rRNA gene amplicon sequencing. Quality-trimmed reads from 45 samples were normalized and assigned to 7514 prokaryotic OTUs using the 97% sequence similarity threshold (see Supplementary File 2). Number of OTUs per sample varied from 792 to 1733 OTUs for the gut samples, and from 187 to 990 OTUs for the nest samples. Gut samples showed a higher richness and diversity than nest samples (Wilcoxon signed-rank tests; richness: $W = 0$, $p < 0.001$; inverse Simpson diversity index: $W = 10$, $p < 0.001$), and only a few OTUs were shared between corresponding pairs of samples (Table S4). Rarefaction curve analysis indicated that the dominant bacterial diversity was captured for most of the tested samples (Fig. S5). An increased sequencing depth would have allowed describing on average 212 ± 49 bacterial OTUs in the case of the gut samples and 183 ± 90 in the case of the nest samples, as indicated by Boneh's estimator implemented in *mothur*.

Bacterial communities of the *C. tuberosus* guts were characterized by the highest average richness (Table S4; Welch two-sample $t$ test; $t = 5.465$, df = 16.003, $p < 0.001$) and diversity (inverse Simpson diversity index; Wilcoxon rank-sum test, $W = 164$, $p < 0.001$) compared to nest builders. In the case of the nest extracts, the diversity and richness were similar between nests inhabited by *C. tuberosus* or by its builder (Wilcoxon rank-sum tests; richness: $W = 33$, $p = 0.395$; inverse Simpson diversity index: $W = 38$, $p = 0.657$). In total, 28 prokaryotic phyla were detected in the gut samples, out of which 13 were represented by at least 100 reads (1% relative abundance) in at least 1 sample. In the case of the nest samples, 30 phyla were identified (12 with at least 1% relative abundance).

Within the gut microbiome, *Spirochaetae* and *Firmicutes* were the most dominant phyla (Fig. 2a). At the family level, the majority of OTUs were classified to *Ruminococcaceae* and *Spirochaetaceae*. In general, similar taxa were present across all gut samples, but their relative abundances varied between termite species. Furthermore, bacterial communities clustered by termite species on the Bray–Curtis-based principal coordinate analysis (PCoA) graph at the OTU level (Fig. 2b), and revealed a dissimilarity higher between hosts (min–max: 0.86–0.97) than between *Cavitermes* and its hosts (0.79–0.93; Wilcoxon signed-rank test; $W = 88$, $p < 0.001$). On average, *Cavitermes* shared over 14.8% ± 2.3 of gut OTUs with its hosts (see Table S5a for details), and the number of shared OTUs was higher between *Cavitermes*

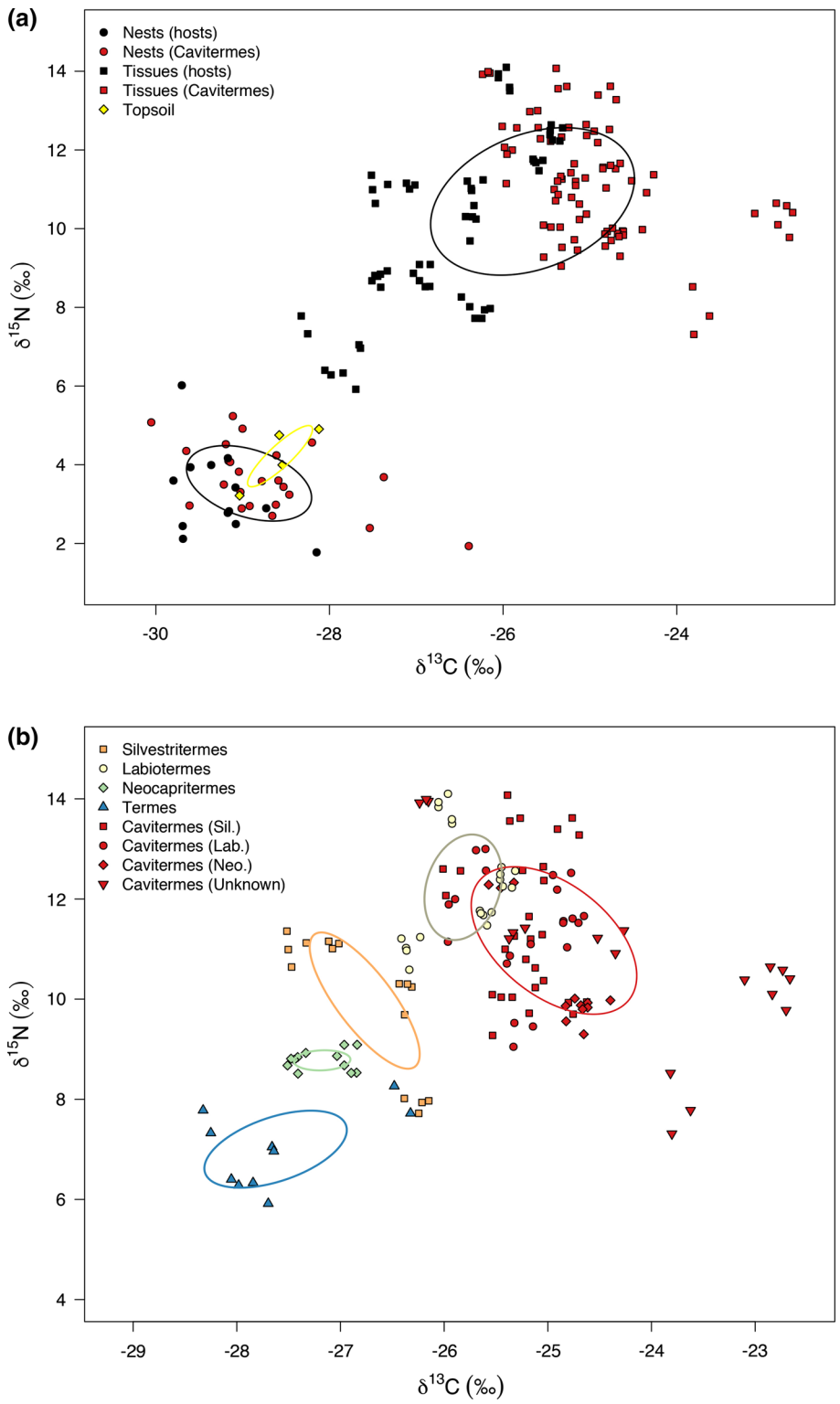


**Fig. 1** Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of **a** the topsoil layer (yellow diamond dots), nests (round dots) and tissues (square dots) from nest builders (black) and *C. tuberosus* (red), with ellipses computed for the topsoil layer, nests and tissues; and **b** tissues of nest builders and *C. tuberosus*, with dot colour and shape representing the termite identity and nest builder, respectively, and ellipses for all species. Ellipses were computed as Bayesian standard ellipses. This figure is available in colour in the online version

and *Labiotermes* (its main host) than with the other termite hosts (Fig. 2c).

In the case of the nest microbiomes, the phylum *Actinobacteria* accounted for an average of 65% ± 15.3 of the relative community abundance (Fig. 3). The majority of the remaining prokaryotic OTUs was assigned to the phylum *Proteobacteria* (on average 23% ± 9.6). On average, 3.8% ± 3.3 of nest OTUs were shared with gut OTUs,

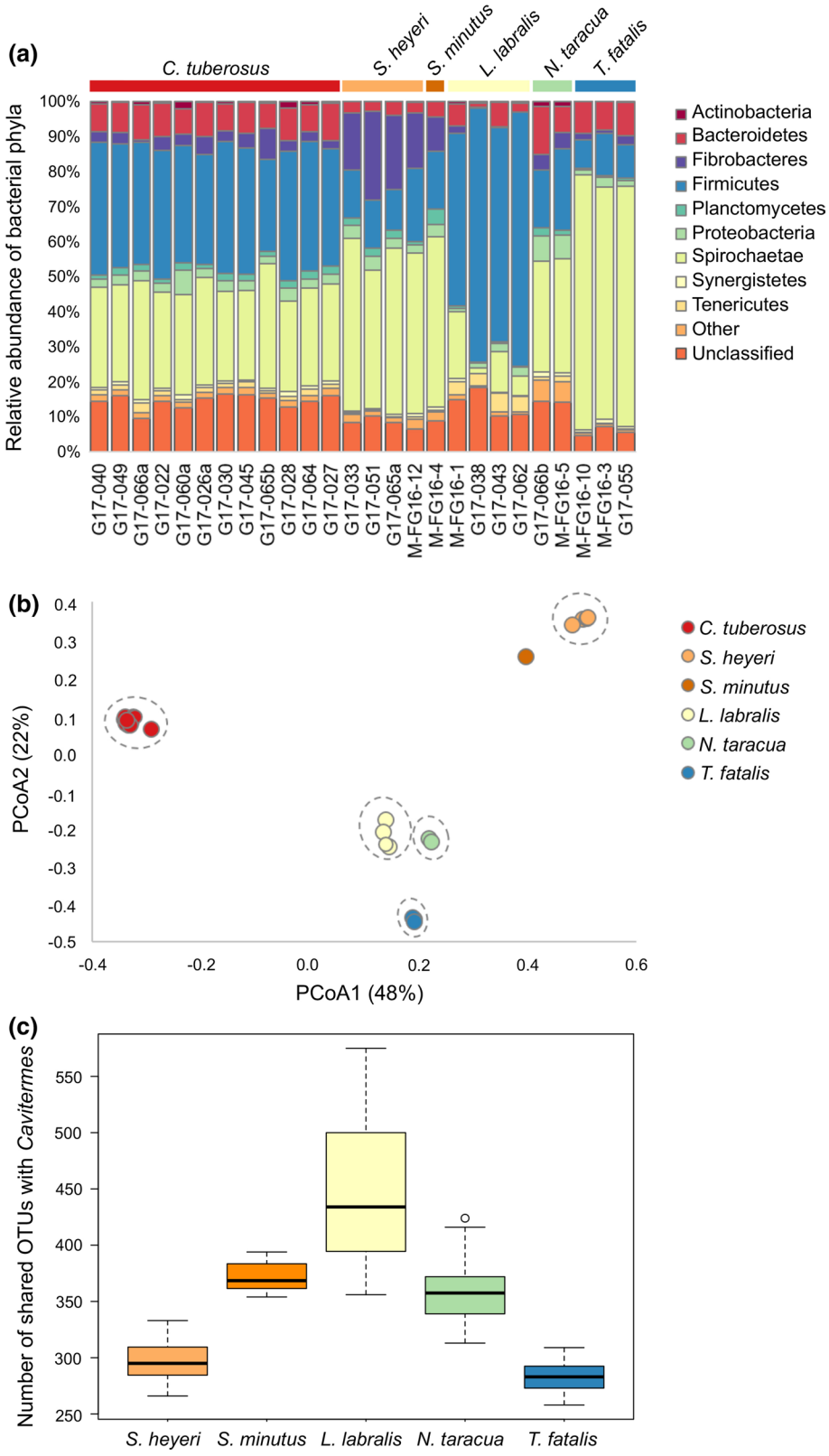


**Fig. 2** Gut community profiles based on the 16S rRNA gene amplicon high-throughput sequencing. **a** Taxonomic distribution of prokaryotic OTUs into major phyla (annotated according to SILVA database *v*128) for *C. tuberosus* and its hosts: *S. heyeri*, *S. minutus*, *L. labralis*, *N. taracua* and *T. fatalis*. Relative abundances were derived based on a number of normalized reads assigned to specific OTUs. **b** Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarities in prokaryotic community structures at the OTU level. **c** Boxplots indicating shared OTUs between *C. tuberosus* and its hosts. This figure is available in colour in the online version

reaching 4.3% ± 2.9 when comparing OTUs from *Cavitermes* guts to OTUs from all nests (see Table S5b for details). Number and proportion of shared OTUs between paired samples of nests and guts are given in Table S4.

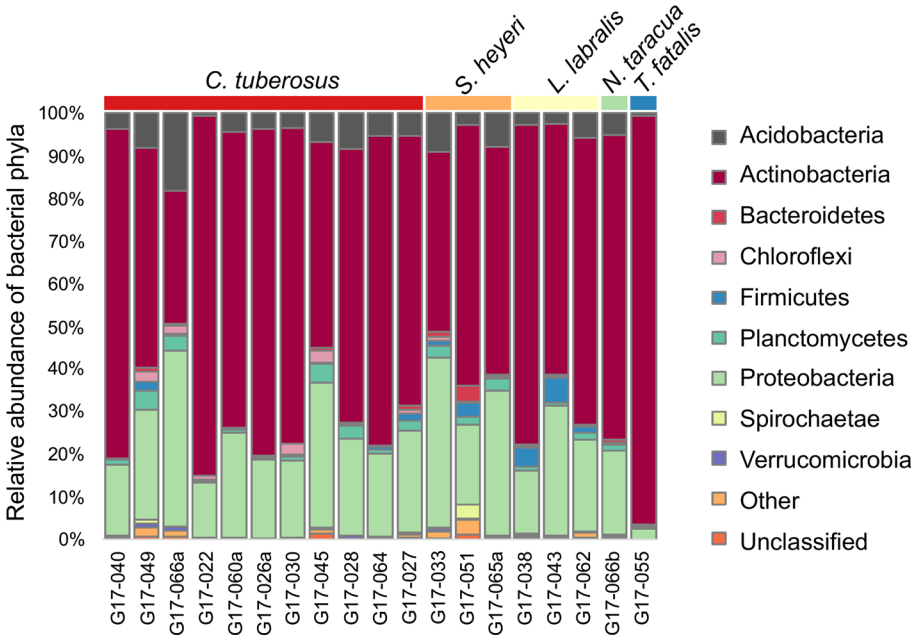


**Fig. 3** Nest community profiles based on the 16S rRNA gene amplicon high-throughput sequencing. Taxonomic distribution of prokaryotic OTUs into major phyla (annotated according to SILVA database *v*128) for *C. tuberosus* and its major hosts: *S. heyeri*, *L. labralis*, *N. taracua* and *T. fatalis*. Relative abundances were derived based on a number of normalized reads assigned to specific OTUs. This figure is available in colour in the online version

### Nitrogen-fixing bacteria in termite guts and nests

By matching the 16S rRNA OTUs from our study to the 16S rRNA genes from the whole prokaryotic RefSeq genomes database, we identified species potentially involved in nitrogen fixation in our samples. As a result, 38 bacterial OTUs were assigned as prokaryotes putatively involved in nitrogen fixation. In general, their relative abundance was higher in the nest microbiomes than in the gut microbiomes (2.33% ± 3.22 vs 0.14% ± 0.10 of all reads; details in Table S4). Nest prokaryotic communities with the presumed nitrogenase activities were mainly dominated by the orders *Burkholderiales*, *Frankiales* and *Rhizobiales*. In the gut microbiomes, lineage I of *Elusimicrobia* constituted the majority.

## Discussion

In the present work, we characterized some dimensions of the ecological niche of the generalist inquiline *C. tuberosus*. Our results show that (1) nests inhabited by *C. tuberosus* display a different physico-chemical composition when compared to nests inhabited by its builder alone; (2) *C. tuberosus* exhibits the broadest range of values for both isotope ratios, as well as the highest ones compared to all other species; (3) its gut microbiota is the most diverse, and a significant proportion of OTUs is shared with its hosts; and (4) nest microbiota harbours a small proportion of putative nitrogen fixers.

### Nutrient-rich arboreal nests

Our results show that nests are overall rich in nutrients, and also indicate that the two categories differ in their composition: nests secondarily colonized by *C. tuberosus* are significantly enriched in some elements (Al, Cd, Co, Cr, Cu and Fe). Organic matter content (OM) is similar between host-inhabited and secondarily colonized nests (Table S6), and the C:N ratio of nests did not differ. A previous study in French Guiana indicated that abandoned termite nests were richer in OM and elements compared with bare soil, making them attractive for secondary occupancy (Bourguignon et al. 2015a). However, studies on mound-building termites from Brazilian Cerrado showed that nests inhabited by primary termites were richer in elements and OM than secondarily colonized nests, and that some nutrients (e.g., nitrate) leach from the latter (Rückamp et al. 2009, 2012). This observation is in contrast to the higher concentration of elements found in the arboreal nests inhabited by *C. tuberosus*. This can be explained by the fact that *C. tuberosus* often colonizes the nest when it is still occupied by its builder, which keeps on bringing further material to the nest. Alternatively, some nutrients in recalcitrant forms may become bioavailable—and therefore detectable by the methods used here—after passage through the gut of inquilines (Bottinelli et al. 2015; Jouquet et al. 2016), or the relative proportions of elements might be altered by the leaching of mobile elements or soil components (OM). Furthermore, contrary to mounds, arboreal nests are protected from leaching by rain-shedding structures (Emerson 1956; Wood 1988), so that they may be less prone to elements runoff.

## Isotopic compositions of nests and tissues

Our data show that isotopic spaces differed between species, sometimes in both dimensions (Fig. 1b), and were consistent with previous measurements from the same study site (Bourguignon et al. 2011). Co-occurring soil-feeding species usually exhibit different isotopic values (Bourguignon et al. 2009, 2011), and this is also the case for species co-habiting within a single mound, i.e., for the builder and the inquiline (Florencio et al. 2013; Fig. S3). Distinct isotopic niches would indicate that species feed on distinct components of the soil and thereby avoid competition.

Termites selectively use soil particles to build different parts of their nests, as shown by different C and N content with presumably different isotopic values (Jouquet et al. 2002, 2015). Our data show that isotopic ratios differ between nest samples and termite tissues (Fig. 1a). In the case of soil-feeding termites, isotopic ratios of both nests and the surrounding soil are highly similar (Tayasu et al. 1997), which is also evidenced by our data (Fig. 1a). However, there is an increase of approximately 5–9‰ and 2–3‰ for $\delta^{15}$N and $\delta^{13}$C, respectively, in tissues (Tayasu et al. 1997). Enrichment in $^{13}$C or $^{15}$N is generally explained by isotopic fractionation during animal and microbial processing of organic compounds during the decomposition processes (Potapov et al. 2019). In termites, differences may further arise from the selective consumption of small soil particles richer in organic matter (characterized by higher $\delta^{15}$N values) and/or their associated microorganisms, as well as from the activity of gut bacterial communities (Nadelhoffer and Fry 1994; Tayasu et al. 1997; Hyodo et al. 2008; Potapov et al. 2019).

Interestingly, the inquiline *C. tuberosus* exhibited higher $\delta^{13}$C and $\delta^{15}$N values compared to all other species. This indicates that they feed on either a distinct diet, or consumed already processed, more humified organic matter and their associated microorganisms (Hyodo et al. 2008; Potapov et al. 2019). Furthermore, this species displayed the largest niche on both isotope ratios, confirming previous observations (Bourguignon et al. 2011). For a given species, variability in isotopic values might relate to the variety of decomposition states of the organic matter in the soil (Bourguignon et al. 2009, 2011). In addition, our results show that nests inhabited by *C. tuberosus* are significantly enriched in $^{13}$C and marginally in $^{15}$N (Table S5), and that both isotopic ratios correlated with those of the corresponding tissue samples on their whole range. These results are consistent with a further degradation/humification of soil organic matter, indicating that nests, in various states of decomposition, may be the main food source of this insect (Peterson and Fry 1987; McCutchan et al. 2003; Hood-Nowotny and Knols 2007; Tiunov 2007; Hyodo et al. 2008; Potapov et al. 2019). Furthermore, some samples of *C. tuberosus* with the highest $\delta^{13}$C values (from nests G17-077, G17B-100 and G17B-138; Fig. 1b; labelled in Fig. S4) showed lower $\delta^{15}$N values, which is compatible with nitrogen fixation by microorganisms (Peterson and Fry 1987; see below). Altogether, these results indicate that *C. tuberosus* feeds on reprocessed organic matter and is a generalist nest feeder.

## Gut microbiota composition of *C. tuberosus* and its hosts

Gut bacteria deeply shaped the ecology and evolution of their hosts by being involved in several core processes such as food digestion and nutrient supplementation, the normal development of the intestine and the immune system, the protection against pathogens and the degradation of toxins, and even host behaviour and social interactions (Douglas 2015; Macke et al. 2017). Gut microbiota encompasses (1) the core microbiota, which are host-specific microbes assembled from diverse environments, which are under host genetic and immune control, as well as (2) a flexible pool of microbes, whose diversity and abundance are modulated by environmental diversity and external conditions (Moran and Sloan 2015; Tai et al. 2015; Shapira 2016).

Diet and gut microenvironment are the primary determinants of bacterial communities and changes in the relative abundance reflect adaptive mechanisms for dietary fluctuations (Abdul Rahman et al. 2015; Mikaelyan et al. 2015, 2017; Waidele et al. 2017). Accordingly, our results show that microbiota differ between species but are similar between colonies of the same species (Fig. 2). Interestingly, the gut microbiota of *C. tuberosus* (Termitinae) is also the most diverse compared to the ones of its hosts and it shares the highest number of OTUs with its main host, *L. labralis* (Syntermitinae), despite their phylogenetic remoteness (Fig. 2b, c). While contrary to endosymbionts, vertical transmission of gut microbiota is generally imperfect (Macke et al. 2017), it is different in termites where the exchange of stomodeal and proctodeal fluids among nestmates (trophallaxis) is the common rule (Nalepa et al. 2001). Also, recent phylogenetic studies on bacterial lineages evidenced rampant horizontal transfer over evolutionary time between termite hosts: gut microbiota is both transmitted vertically colony-to-offspring and horizontally through colony-to-colony transfer (Bourguignon et al. 2018). Horizontal transfer between species could occur through heterospecific cannibalism after agonistic encounters or through the consumption of nest material (Thorne and Haverty 1991; Nalepa et al. 2001). This suggests that the gut microbiota of *C. tuberosus* have been influenced by the gut communities of its hosts, and may be adapted to digest nest materials built by several species.

## Nest-associated bacterial communities

After gut passage, the soil organic matter is transformed into organo-mineral micro-aggregates incorporated as faeces in the building of the nest, and these nutrient-rich substrates facilitate the growth of microorganisms (Garnier-Sillam et al. 1985; Brauman 2000). Our results show that bacterial communities are drastically different between termite gut and nests, and that members of the phylum *Actinobacteria* dominate in the latter (Fig. 3; 1 vs 65% in relative abundance). *Actinobacteria*-dominated bacterial communities were also found in mounds of the soil-feeder *Cubitermes niokoloensis*; lineages from mounds differed from the surrounding soil and were more diverse (Fall et al. 2004, 2007). As a matter of fact, bacterial communities usually strongly differ between the termite gut, the nest and the surrounding soil (Ohkuma and Brune 2011; Manjula et al. 2016).

While the diet of wood-feeding termites consists of carbon-rich but nitrogen-poor food, soil feeders should not be limited by nitrogen (Waller and La Fage 1987; Brune and Ohkuma 2011). Nitrogen requirements of the latter are covered by the mineralization of peptides, amino sugars and microbial biomass present in the humus (Ji and Brune 2006; Brune and Dietrich 2015). This mineralization is evidenced by huge concentrations of ammonium in the faeces, which serve as a basis for nest building and protect this nitrogen form from leaching through adsorption onto the nest material (Ji and Brune 2006). However, in the case of secondary colonization of an arboreal nest, no further material is brought to the nest, and nitrogen supply may become limiting at some point. In termites, nitrogen economy can be achieved through controlled cannibalism of nestmates and the recycling of uric acid (Moore 1969). While this latter pathway is important in wood-feeding termites, its significance in soil feeders is not known (Potrikus and Breznak 1980, 1981; Brune and Ohkuma 2011; Thong-On et al. 2012). Preliminary data showed that transcripts of the gene encoding for urease, an enzyme involved in the catabolic pathway of uric acid (into ammonia), are detected in gut samples of several neotropical soil-feeding termites (M.M. and M.C., unpublished data), suggesting that this pathway for nitrogen economy may not be restricted to wood feeders.

Beside recycling pathways discussed above, one major feature of the symbiosis between termites and gut microorganisms is the fixation of atmospheric nitrogen (Tayasu et al. 1994). The gene *nifH* (encoding for the dinitrogenase reductase) is the key gene involved in this process (Ohkuma et al. 1999). Such a process may also occur in the nest material, and could be of importance in arboreal nests, to which no organic matter is brought when the colony of the nest-building species vanishes. Based on our analyses, potential nitrogen-fixing bacteria represent at least 2.33% of the community abundance in nest samples. However, our analysis is by no means exhaustive as it is based on published genomes and the quality of annotations. Nevertheless, our results suggest that a few bacteria in the nest might fix atmospheric nitrogen. Such a process would compensate for the consumption of nest material, a hypothesis which is further supported by the similarity of the C:N ratio between A and B nests. However, the low concentration of the micronutrient molybdenum (30 µg $kg^{-1}$ in our nest samples; see Table S6), an essential cofactor for some nitrogenases (Eady 1996), could be limiting as in other lowland tropical forests (Barron et al. 2009). Therefore, nitrogen fixation activity and its intensity remain to be firmly demonstrated through biological assays.

## Generalist inquiline and ecological success

*Cavitermes tuberosus* colonizes nests of several arboreal termite species and can be considered as a broad-spectrum inquiline. In this study, we demonstrate that (1) this species feeds on the nest material and consumes already processed, more humified, nest organic matter; (2) its gut microbiota is much more diverse than that of its hosts, and probably adapted to digest nest materials from several species; and (3) a few bacteria in the nest might be able to fix atmospheric nitrogen, a process that would balance the consumption of the nest material. Furthermore, the endosymbiotic bacterium *Wolbachia* was recently shown to inhabit a bacteriome in the anterior part of the mesenteron of *C. tuberosus* to which it may supply essential nutrients (Hellemans et al. 2019b). Altogether, these features explain the ecological success of this inquiline, despite its inability to build its own nest.

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**Author contribution statement** SH and YR designed the study. SH, MM, DF, and YR collected the material. SH and TD performed soil analyses; SH and GL performed isotopic analyses; and MM and MC performed Illumina sequencing and subsequent analyses. All authors contributed significantly to the manuscript and approved the final version.

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## Compliance with ethical standards

**Conflict of interest** We declare we have no competing interests.

**Data archiving** Sequences produced for this study have been deposited in GenBank repository under Project accession KBWO01000000 (see details in Supplementary Table S2).

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