



Vertical transmission of cellulolytic protists in termites is imperfect, but sufficient, due to biparental transmission

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Abstract

Many host-symbiont relationships are maintained through vertical transmission. While maternal symbiont transmission is common, biparental transmission is relatively rare. Protist-dependent termites are eusocial insects that harbor obligate, cellulolytic protists in their hindguts. Protists are vertically transmitted by winged reproductives (alates), which disperse to biparentally establish new colonies. Vertical transmission in protist-dependent termites is imperfect, as the protist communities of alates are often incomplete. Biparental transmission of protists may make it unnecessary for alates to harbor complete communities, as colonies would acquire symbionts from both founding kings and queens, which together may harbor sufficient inoculums. To investigate this hypothesis, the protist communities of *Coptotermes gestroi* and *C. formosanus* alates and colonies were examined using 18S rRNA amplicon sequencing. The complete protist communities of these *Coptotermes* species are composed of five parabasalid species each. Whereas alates often harbored 1–3 protist species, nearly all colonies harbored 4–5 species, implying biparental transmission. The probability of each protist species being present in at least one founding alate was used to determine expected protist occurrence in colonies. For most protists, expected and observed occurrence did not significantly differ, suggesting that each protist species only needs to be harbored by one founding alate to be acquired by colonies. Our results imply that biparental transmission allows founding reproductives to transmit adequate symbiont communities to colonies despite their individual communities being incomplete. We discuss biparental transmission in protist-dependent termites in the context of other biparentally transmitted symbioses.

Keywords Subterranean termites · Flagellates · Microbiome · Protozoa · Social insects

1 Introduction

Symbiotic relationships range from purely facultative to strictly obligate (Kearns et al. 1998; Delabie 2001; Fisher et al. 2017). Symbioses characterized by partners in persistent contact are often relationships between multicellular hosts and their associated microbial symbionts (Bright and Bulgheresi 2010). Obligate symbionts often provide essential nutrients to their hosts (Douglas 2016; Skidmore and Hansen 2017). Such host-symbiont relationships are maintained through symbiont transmission (Bright and Bulgheresi 2010). Conventionally, transmission is described as occurring either horizontally or vertically, although some hosts can acquire symbionts through both transmission modes, i.e., mixed-mode transmission (Ebert 2013; Russell 2019).

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Horizontal transmission usually refers to symbiont acquisition from an environmental source of free-living microbes (Bright and Bulgheresi 2010). This term is also used to describe both intraspecific and interspecific transmission among hosts, i.e., host switching (Chrostek et al. 2017; Bourguignon et al. 2018). In contrast, vertical transmission refers to offspring acquiring symbionts from one or both parents (Bright and Bulgheresi 2010). For example, insect hosts vertically transmit symbionts through trans-ovarial transmission (Douglas 1998; Dan et al. 2017; Russell et al. 2019) or other mechanisms, such as milk gland secretions in tsetse flies (Balmand et al. 2013) and symbiont capsules in plataspid stinkbugs (Fukatsu and Hosokawa 2002). Whereas maternal symbiont transmission is common, biparental transmission is comparatively rare. Nearly all described examples of biparental transmission are characterized by offspring potentially acquiring both maternal and paternal lineages of facultative bacterial symbionts, which progeny usually sufficiently obtain through solely maternal symbiont transmission (Moran and Dunbar 2006; Damiani et al. 2008; Watanabe et al. 2014; De Vooght et al. 2015). Unlike these examples, biparental transmission in *Aporrectodea tuberculata*, a lumbricid earthworm species, is probably not facultative, as offspring likely often require *Verminephrobacter* lineages from both hermaphroditic parents to acquire adequate inoculums (Paz et al. 2017). Like lumbricid earthworms, eusocial cockroaches, commonly known as termites, may also be an example of non-facultative biparental symbiont transmission.

Termites are ecologically dominant social insects (Engel et al. 2009), which are important decomposers of dead plant material in tropical (Griffiths et al. 2019), subtropical (Stoklosa et al. 2016), savanna (Collins 1981), and desert ecosystems (Whitford et al. 1982). Globally, their biomass is comparable to that of ants (Bar-On et al. 2018; Eggleton 2020). Termites live in organized social units known as colonies, which are composed of three castes: workers, soldiers, and reproductives. All termites are eusocial, as they exhibit overlapping generations, cooperative brood care, and reproductive division of labor (Wilson 1971). The queen and king of a colony are generally the only individuals that reproduce (Eggleton 2011). Alates are winged reproductives (i.e., potential queens and kings) that disperse from their natal colony to establish new ones (Nutting 1969). Workers perform essential colony tasks, including foraging, tending eggs and larvae, feeding soldiers and reproductives, and building and maintaining colony architecture (Krishna 1969). Soldiers, in concert with colony architecture, defend against predators, such as insectivorous mammals and particularly ants (Noirot and Darlington 2000; Eggleton 2011).

Conventionally, termites are considered to belong to one of two groups depending upon their symbionts. The

protist-dependent termites (historically called the lower termites) harbor both prokaryotic symbionts and cellulolytic protists. This group is comprised of all termite families except Termitidae. In contrast, termites that belong to Termitidae (historically called the higher termites) solely harbor symbiotic prokaryotes (Brune and Dietrich 2015). For this study, we focused on protist-dependent termites and their obligate, wood-digesting protist symbionts (Cleveland 1923, 1924).

Termite protists belong to either the phylum Parabasalia or the order Oxymonadida (phylum Preaxostyla). Protists harbored by termites reside primarily in an anterior, dilated region of the hindgut known as the hindgut paunch, but can occur throughout the hindgut, including the rectum (Brune 2014). Most hindgut bacteria and archaea are either intracellular endosymbionts or cell-surface ectosymbionts of protists (Ohkuma and Brune 2011). Hindgut prokaryotes are involved in a variety of metabolic processes, including acetogenesis and nitrogen metabolism (Brune and Ohkuma 2011). In short, protist-dependent termites harbor symbiotic communities of prokaryotes and cellulolytic protists that are fundamental to their biology (Peterson and Scharf 2016).

Hindgut symbionts are transmitted through proctodeal trophallaxis (McMahan 1969; Nalepa 2015). During this behavior, symbiont-rich hindgut fluids voided from the rectum of a donor termite are consumed by a recipient nestmate (McMahan 1969). Unlike many insect hosts, protist-dependent termites must horizontally reacquire symbionts from nestmates after each molt, as termite protists die during this process (Honigberg 1970; Nalepa 2017). They do so by repeatedly consuming proctodeal fluids donated by inter-molt nestmates (Nutting 1956; Nalepa 2015). This behavior is also the mechanism by which vertical transmission occurs in protist-dependent termites (Nalepa et al. 2001).

Vertical transmission in protist-dependent termites begins with newly emerged, last instar nymphs (i.e., the developmental stage that precedes the alate stage) reacquiring symbionts from their nestmates (Cleveland 1925; May 1941; Inagaki et al. 2022). Before dispersing, nymphs molt a final time to reach the alate stage. Unlike other molts, a small symbiont population is retained during the imaginal molt that recolonizes the hindgut after ecdysis (May 1941; Grassé and Noirot 1945; Nutting 1956; Honigberg 1970; Nalepa 2017). After maturation (Nutting 1969), alates disperse to establish new colonies, harboring small inoculums (Cook and Gold 1998; Lewis and Forschler 2004; Shimada et al. 2013; Velenovsky et al. 2021). Before the emergence of their initial workers, the protist abundances of both kings and queens considerably increase from wood consumption (Cleveland 1925; Rosengaus and Traniello 1991; Shimada et al. 2013; Velenovsky et al. 2021). The first workers of colonies then vertically acquire symbionts from presumably

both reproductives through repeated proctodeal trophallaxis (Shellman-Reeve 1990; Nalepa 2015; Brossette et al. 2019).

With rare exception (Taerum et al. 2018), each protist-dependent termite species harbors a host-specific community of cellulolytic protists that is generally consistent throughout its geographic range (Kirby 1937; Honigberg 1970; De Martini et al. 2021). However, colonies sometimes lack one or more characteristic protist species (Kitade and Matsumoto 1993; Kitade et al. 2012; Taerum et al. 2018; Michaud et al. 2020; De Martini et al. 2021). This is likely attributable to some colonies being established by reproductives with incomplete protist communities (Honigberg 1970). In agreement with this notion, alates sometimes do not harbor all of their characteristic protists (Lewis and Forschler 2004; Michaud et al. 2020). In short, unlike many other hosts, vertical transmission in protist-dependent termites is imperfect (Michaud et al. 2020).

How protist-dependent termites and their obligate, cellulolytic protists maintain their relationships through imperfect vertical transmission is largely unclear. Alates sometimes harboring incomplete communities implies that they have not evolved a mechanism that ensures they disperse with all of their characteristic protists. Biparental colony establishment may make it unnecessary for alates to harbor complete protist communities, as the initial workers of colonies can potentially acquire symbionts from both founding reproductives, which collectively may harbor sufficient inoculums.

To test this hypothesis, we investigated the protist communities of *Coptotermes gestroi* and *C. formosanus* alates and colonies through 18S rRNA amplicon sequencing. We determined if conspecific male and female *Coptotermes* alates harbor similar protist communities. In addition, we ascertained whether the protist communities of *Coptotermes* alates and colonies differ with regard to species richness. Finally, we calculated the probability of each protist species being harbored by at least one founding alate during colony establishment and compared these probabilities to data from *Coptotermes* colonies. Our results imply that biparental transmission allows newly established colonies to acquire

sufficient inoculums despite founding reproductives often individually harboring incomplete protist communities.

2 Materials and methods

2.1 Alate collection and colony establishment

Coptotermes gestroi and *C. formosanus* alates were collected at a single, private residence in Ft. Lauderdale, FL (26.105°N, 80.175°W) during their 2014–2019 dispersal flight seasons using a light trap (Chouvenc et al. 2015). Collected alates were kept in a plastic box that contained moist corrugated cardboard until the following morning when they were used to establish colonies. The species and sex of dealates were determined using morphological characters (Weesner 1969; Su et al. 1997). Conspecific colonies were established by introducing dealate pairs into individual rearing-units. Each rearing-unit consisted of a transparent plastic cylindrical vial (8 cm × 2.5 cm diameter, internal volume = 37 mL, Fisher Scientific, Pittsburgh, PA) that contained moistened organic soil, *Picea* sp. wooden blocks, and 3% agar solution to maintain moisture (Chouvenc et al. 2015). The lids of rearing-units were punctured with a safety pin to allow air exchange while still preventing escapees. As colonies grew, they were first transferred to 17.15 cm × 12.22 cm × 6.03 cm transparent plastic containers (Pioneer Plastics, Dixon, KY). Later, colonies that grew beyond the capacity of their containers were transferred to larger 45.72 cm × 30.48 cm × 15.24 cm transparent plastic containers (Carlisle FoodService Products, Oklahoma City, OK). Colonies were sprayed with deionized water and provisioned with appropriately sized *Picea* sp. wooden blocks as needed. All colonies were stored at 28 ± 1°C and approximately 80% humidity.

2.2 Sampling scheme

Table 1 contains the numbers of *C. gestroi* and *C. formosanus* colonies that were sampled for each age. In total, 46 *C. gestroi* and 37 *C. formosanus* colonies were sampled. We investigated both *C. gestroi* and *C. formosanus* to confirm that our results were not species-specific. For 1–6-month-old colonies, up to five workers, five soldiers, the king, and the queen were sampled from each colony, depending upon which individuals were present at the time. For 1.5–3.5-year-old colonies, between three to eight workers were sampled from each colony. The numbers of male and female *C. gestroi* and *C. formosanus* alates that were sampled are shown within Table 1. In sum, 24 *C. gestroi* and 47 *C. formosanus* alates were sampled. Differential

Table 1 The numbers of *C. gestroi* and *C. formosanus* alates and colonies sampled

Age	<i>C. gestroi</i>	<i>C. formosanus</i>
Male alates	14	26
Female alates	10	21
1-month-old	3	3
2-month-old	3	3
3-month-old	3	3
4-month-old	3	3
5-month-old	3	3
6-month-old	3	3
1.5-year-old	18	11
2.5-year-old	4	3
3.5-year-old	6	5

availability of biological material during sampling resulted in unequal sample sizes in some instances (Table 1).

2.3 18S rRNA amplicon sequencing

The complete protist community of *C. gestroi* consists of five parabasalid species: *Pseudotrichonympha leei*, *Holomastigotoides batututi*, *H. bigfooti*, *Cononympha skunkapei*, and *Con. monstrummogolloni* (del Campo et al. 2017; Jasso-Selles et al. 2020). Similarly, the complete community of *C. formosanus* is also comprised of five parabasalids: *P. grassii*, *H. hartmanni*, *H. minor*, *Con. leidy*, and *Con. koidzumii* (Koidzumi 1921; Jasso-Selles et al. 2020; Nishimura et al. 2020). Hindguts were removed from termites by grasping the thorax with forceps and pulling on the posterior abdominal segments with fine-tipped forceps (Lewis and Forschler 2004). The MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI) was used to extract DNA from dissected hindguts following the manufacturer's protocol.

Dual-indexed amplicon libraries were created via a two-step PCR procedure (Kozich et al. 2013). Primers specific to 18S rRNA genes of parabasalids with Illumina adaptor sequences at their 5' ends—nexF-ParaV45F 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3' and nexF-ParaV45R 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3'—were used for the first PCR (Jasso-Selles et al. 2020; De Martini et al. 2021). Indexing barcodes were attached to both ends of each amplicon during the second PCR (Hamady et al. 2008). Conditions for the first PCR were as follows: 3 min denaturation at 95°C, 30 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 50 s, and a final 10 min extension at 72°C. Conditions for the second PCR were as follows: 3 min denaturation at 95°C, 8 cycles of 95°C for 30 s, 50°C for 30 s, and 68°C for 50 s, and a final 10 min extension at 68°C. Reactions for the first PCR used 12.5 µL of EconoTaq PLUS GREEN 2X Master Mix (Lucigen), 2.5 µL of 10 µM forward and reverse primers, and 2 µL of template DNA in 25 µL reactions. Reactions for the second PCR used 12.5 µL of EconoTaq PLUS GREEN 2X Master Mix, 2.5 µL of combined forward and reverse primers (2 µM), and 2.5 µL of first PCR products in 25 µL reactions. AMPure XP beads in concert with a Biomek NX^P Automated Workstation (Beckman Coulter Life Sciences, Indianapolis, IN) were used for PCR purification. PCR products were quantified using the Qubit 1X dsDNA BR Assay Kit (Invitrogen, Waltham, MA) and a Synergy HT Microplate Reader (BioTek, Winooski, VT). An approximately equal quantity of DNA (ng) from each PCR reaction (i.e., each individually sampled termite) was used for pooling. Samples were sequenced using 2 × 300 bp paired-end sequencing on the Illumina MiSeq System.

Demultiplexed FASTQ files were submitted to NCBI Sequence Read Archive under BioProject accession number PRJNA611820. Amplicon data from *C. gestroi* and *C. formosanus* workers from 1.5–3.5-year-old colonies were used previously (Jasso-Selles et al. 2020). For this study, those data were analyzed differently as described below.

2.4 Amplicon sequence analysis

The bioinformatics platform QIIME 2TM 2020.8 was used to analyze amplicon sequences (Bolyen et al. 2019). Demultiplexed FASTQ files (Casava 1.8 paired-end) were imported using 'qiime tools import'. Data from each sequencing run were filtered, trimmed, denoised, and merged separately using DADA2 (Callahan et al. 2016). DADA2 was also used to remove chimeric sequences. A Naive Bayes classifier was trained on a reference file that consisted of 1,075 18S rRNA gene sequences from parabasalids and oxymonads using 'qiime feature-classifier' (Bokulich et al. 2018; Jasso-Selles et al. 2020; De Martini et al. 2021). Amplicon sequence variants from each sequencing run were taxonomically classified separately with this classifier using 'qiime feature-classifier'. Any *C. gestroi* samples with reads from more than one *C. formosanus* protist or that had less than 95% of their reads classified as *C. gestroi* protists were discarded. For *C. formosanus*, any samples that had less than 95% of their reads classified as *C. formosanus* protists or with reads from more than one *C. gestroi* protist were discarded. Samples with less than 70 total reads were also discarded. In sum, 488 samples (i.e., individual termites) comprised the final dataset, of which 242 were *C. gestroi* samples and 246 were *C. formosanus* samples. The mean read abundance of samples was ≈17,500 merged reads. In addition, 95% (463/488) of samples had at least 900 reads from the parabasalids of interest (Supplementary Tables S1–S4).

2.5 Protist presence/absence

The abundances of protists in samples varied widely (Supplementary Tables S1–S4). Because 18S read abundances of *C. gestroi* and *C. formosanus* protists do not accurately reflect cellular abundances (Jasso-Selles et al. 2020), they were not investigated, except to assess the read depths of alate samples (see below for details). For all other analyses, we coded each protist species as being either present in or absent from each sample. For *Holomastigotoides* and *Cononympha*, a protist species was considered present in a sample if the sample included one or more reads that were classified as that species. For *Pseudotrichonympha*, a protist species was considered present in a sample if 100 or more reads were classified as that species. This was an additional

means of quality control that was used to account for possible index hopping. For *Pseudotrichonympha*, we occasionally observed (12.5% of samples) between-species index hopping, i.e., *C. gestroi* samples with *P. grassii* reads or *C. formosanus* samples with *P. leei* reads. Nearly all (90.2%) of these instances of between-species index hopping involved less than 100 misidentified reads. Therefore, we chose to account for the possibility of within-species index hopping by using a 100-read-threshold for *Pseudotrichonympha* species. In contrast, between-species index hopping for *Holomastigotoides* and *Cononympha* species was rarely observed (1.6% of samples).

Data from individual termites were used to infer the protist communities of colonies. If a protist species was deemed present in at least one individual from a colony, then that species was considered part of the protist community of the colony. Therefore, a protist species was only considered absent from a colony, if all individuals from the colony lacked that species. The protist communities of colonies were frequently apparent, as nestmates often harbored similar communities (Supplementary Tables S1–S2).

In all likelihood, using amplicon data from all individuals from a colony to infer a protist species' absence greatly reduced the chance of incorrectly inferring absence. In contrast, the chance of incorrectly inferring a protist species' absence from an alate may have been somewhat higher, as protist species were deemed present in or absent from alates based solely upon amplicon data from each individual alate hindgut, which varied in total read abundance. For this reason, we chose to assess the possibility that insufficient sequencing may have affected our alate results. To do so, we computed the proportional-read-abundance of each protist species in each alate sample using the following formula: [total reads from protist species / total reads from all protists]. Using these values, we determined the minimum proportional-read-abundance for each protist species (Supplementary Tables S3–S4). We chose to use the minimum value, rather than the mean or median value, because we considered it to be a reasonably conservative, empirical threshold for assessing the sequencing depths of alate samples. From the observed minimum proportional-read-abundance values, we computed the expected number of reads needed to detect one read from each protist species using the following formula: $[1 / \text{minimum proportional-read-abundance}]$ (Supplementary Tables S3–S4). Among *C. gestroi* alates, the lowest proportional-read-abundance of any protist species, when present, was 0.22% of reads, which was observed in *H. batututi*. Therefore, the expected number of reads needed to detect one read from *H. batututi* was calculated as '455' reads. The read depth of only one *C. gestroi* alate fell below this threshold, so we concluded that insufficient sequencing likely did not affect our

C. gestroi results. Among *C. formosanus* alates, the lowest proportional-read-abundance of any protist species, when present, was 0.15% of reads, which was observed in *Con. koidzumii*. Thus, the expected number of reads needed to detect one read from *Con. koidzumii* was calculated as '667' reads. In this case, nine *C. formosanus* alates fell below this threshold, so, in addition to performing the *C. formosanus* statistical analyses using the complete alate dataset, we also chose to perform them using a reduced dataset that did not include the nine alates with read depths below '667' reads. With regard to statistical significance, the results from both datasets were identical. Therefore, only the results from the statistical analyses that used the complete *C. formosanus* alate dataset are presented.

2.6 Statistical analyses

For both *Coptotermes* species, data from differently aged colonies were pooled together for analyses. These poolings were justified because the protist communities of all sampled colonies, regardless of age, originated from their founding alate pairs (Nutting 1969; Honigberg 1970; Michaud et al. 2020). Additionally, the richness values of the protist communities (see below) of incipient (1–6-month-old) and juvenile (1.5–3.5-year-old) *C. gestroi* (Mann-Whitney $U=219.5$; $P=0.4272$) and *C. formosanus* (Mann-Whitney $U=119.5$; $P=0.0710$) colonies did not significantly differ, further justifying these poolings. Comparisons between *Coptotermes* species were not performed because they were not a focus of our study.

The protist community richness (i.e., the number of protist species harbored) was calculated for each colony and alate. To determine if the community richness values for male and female alates significantly differed, a Mann-Whitney U test was performed for each *Coptotermes* species through the 'wilcox.test' function within the R package 'stats'. Likewise, a Mann-Whitney U test was also separately performed for *C. gestroi* and *C. formosanus* to determine whether the richness values for colonies and alates (male and female alates combined) significantly differed. These values were also investigated using Fisher's exact tests. For these tests, the community richness of each colony and alate was classified as either 1–3 or 4–5 protist species. To determine if the richness distributions for colonies and alates significantly differed, a Fisher's exact test was performed for each *Coptotermes* species through the 'fisher.test' function of the R package 'stats'.

A Mann-Whitney U test was also performed for each *Coptotermes* species to ascertain whether the protist community richness values for incipient and juvenile colonies significantly differed. The results of these tests (see above)

supported the decision to pool data from differently aged colonies together for analyses.

The protist communities of colonies and alates are examples of a multiple-response categorical variable (MRCV) (Bilder and Loughin 2004), as both colonies and alates can harbor more than one protist species. Therefore, it was inappropriate to use conventional chi-square tests to determine if the species composition distributions for male and female alates significantly differed, i.e., if male and female alates harbored protist species at different frequencies. Instead, a test for multiple marginal independence (MMI) was performed for each *Coptotermes* species (Agresti and Liu 1999; Bilder et al. 2000). To do so, the 'item.response.table' function within the R package 'MRCV' was used to construct summary tables of positive (protist harbored) and negative (protist not harbored) responses observed for male and female alates (Kozioł and Bilder 2014). In other words, a 2×2 marginal table (i.e., similar to a contingency table) with row labels 'male' and 'female' and column labels 'positive' and 'negative' was constructed for each protist species. Using these tables, the 'MI.test' function within 'MRCV' was used to perform tests for MMI through the Bonferroni approach (Agresti and Liu 1999; Kozioł and Bilder 2014). This approach first performs a conventional chi-square test for each marginal table. Then, the *P*-values from these tests are adjusted based upon the total number of tests conducted. If any of the adjusted *P*-values are significant, then the null hypothesis of MMI is rejected (Bilder et al. 2000). The overall *P*-value for a MMI test performed through this approach is equal to the minimum of the observed *P*-values (Agresti and Liu 1999). While the MMI test for *C. formosanus* male and female alates used data from all five protist species, the test for *C. gestroi* only analyzed data from four species. It was unnecessary to analyze data from *Con. skunkapei* because this species was harbored by all male and female alates.

The probability of each protist species being harbored by at least one founding alate was calculated using the following formula: $[1 - [P(\text{species absent from male alate}) \times P(\text{species absent from female alate})]]$. For each protist species, the values of $[P(\text{species absent from male alate})]$ and $[P(\text{species absent from female alate})]$ were derived from the observed data. For example, for *H. batututi*, $[P(\text{species absent from male alate})]$ was calculated as $(1 - 0.357 = 0.643)$. The probabilities of species being present in at least one founding alate were regarded as expected values for the occurrence of protist species in colonies.

To compare expected and observed protist occurrence, distributions of positive (protist harbored) and negative (protist not harbored) expected responses were produced. As described above, these distributions were based upon the calculated probabilities. For example, for *H. batututi*,

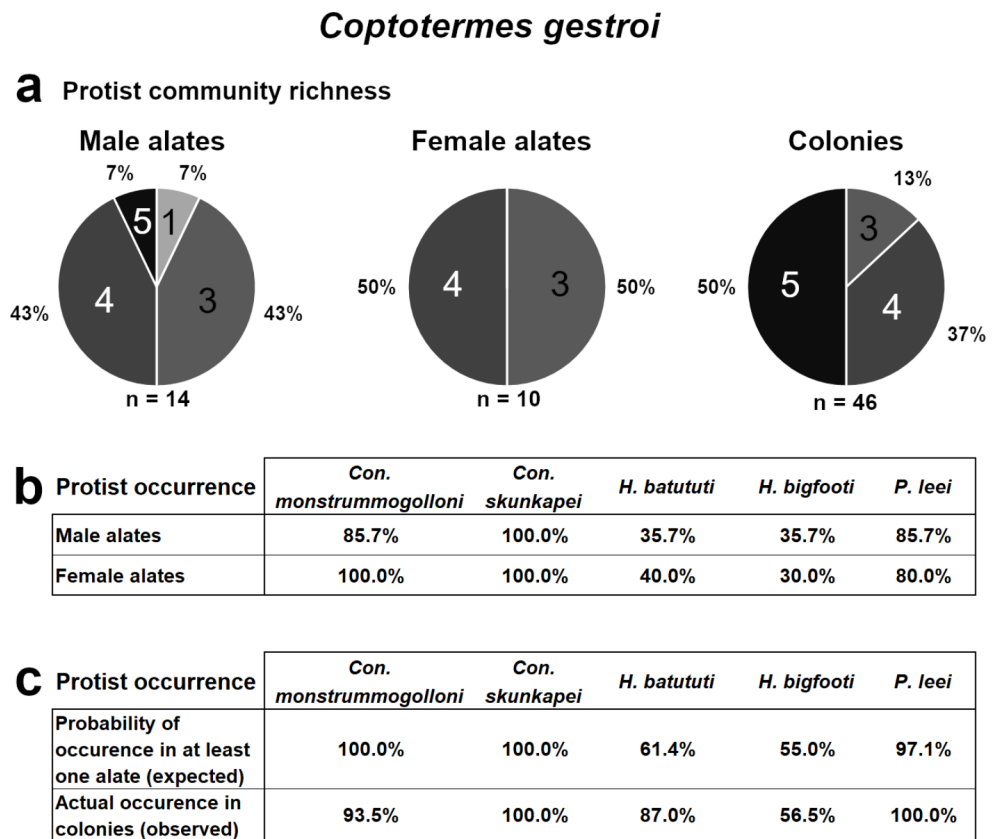
the probability that this species is harbored by at least one founding alate was calculated as '0.614'. Therefore, the expected distribution for *H. batututi* consisted of '28' positive and '18' negative responses, i.e., this species was expected to be present in '28' and absent from '18' colonies. The total number of responses for *C. gestroi* and *C. formosanus* expected distributions was chosen to be '46' and '37', respectively. These numbers were selected to match the numbers of observed colonies. Using these distributions, a summary table of expected and observed responses was constructed for each protist species (Kozioł and Bilder 2014). Tests for MMI were performed using the Bonferroni approach described above (Agresti and Liu 1999; Kozioł and Bilder 2014). The MMI test for *C. formosanus* used data from three protist species, while the test for *C. gestroi* analyzed data from four species. For *C. gestroi*, it was unnecessary to analyze data from *Con. skunkapei*, as all colonies harbored this species and its probability was calculated as '1'. Similarly, it was unnecessary to analyze data from *H. hartmanni* and *H. minor* because all *C. formosanus* colonies harbored these species and their probabilities were calculated as '0.998' and '0.994', respectively. For both species, the expected number of positive responses after rounding (i.e., $(0.998 \times 37 = 36.93)$ and $(0.994 \times 37 = 36.78)$) equaled '37'. Therefore, like *Con. skunkapei*, the expected and observed distributions for these species were identical. All analyses were performed using R version 4.0.5 (R Core Team 2021).

3 Results

3.1 *C. gestroi* male and female alates

The richness values of the protist communities of male alates ranged from 1 to 5 species, whereas female alates harbored 3–4 species (Fig. 1a). However, the values for male and female alates did not significantly differ (Mann-Whitney $U = 70$; $P = 1$). For protist occurrence, the percent of female *C. gestroi* alates ($n = 10$) that harbored each protist species ranged from 30% for *H. bigfooti* to 100% for both *Cononympha* species (Fig. 1b). Similarly, between 35.7% (both *Holomastigotoides* species) and 100% (*Con. skunkapei*) of male *C. gestroi* alates ($n = 14$) harbored each protist species (Fig. 1b). The species composition distributions for male and female alates were not significantly different (MMI test; $X^2 = 0.59$; $df = 1$; $P = 1$; *Con. monstremoggolloni* chi-square test). The results of the other three chi-square tests conducted for the MMI test were as follows: *H. batututi* ($X^2 = 0.05$; $df = 1$; $P = 1$), *H. bigfooti* ($X^2 = 0.09$; $df = 1$; $P = 1$), and *P. leei* ($X^2 = 0.14$; $df = 1$; $P = 1$).

Fig. 1 The results observed for *C. gestroi* alates and colonies. (a) The numbers of protist species harbored by male alates, female alates, and colonies. (b) The percents of male and female alates that harbored each protist species. (c) The percent of colonies that harbored each protist species, and the probability of each species being harbored by at least one founding alate



3.2 *C. formosanus* male and female alates

Male alates harbored 1–5 protist species, while the communities of female alates were composed of 1–4 species (Fig. 2a). Like *C. gestroi*, there was no significant difference between the richness values for male and female alates (Mann-Whitney $U=244$; $P=0.5081$). For protist occurrence, between 0% (*Con. leidy*) and 95.2% (both *Holomastigotoides* species) of female *C. formosanus* alates ($n=21$) harbored each protist species (Fig. 2b). Likewise, the percent of male *C. formosanus* alates ($n=28$) that harbored each protist species ranged from 3.8% for *Con. leidy* to 96.2% for *H. hartmanni* (Fig. 2b). Like *C. gestroi*, the species composition distributions for male and female alates did not significantly differ (MMI test; $X^2=3.15$; $df=1$; $P=0.3793$; *P. grassii* chi-square test). The results of the other four chi-square tests performed for the MMI test were as follows: *H. hartmanni* ($X^2=0.02$; $df=1$; $P=1$), *H. minor* ($X^2=0.69$; $df=1$; $P=1$), *Con. koidzumii* ($X^2=0.20$; $df=1$; $P=1$), and *Con. leidy* ($X^2=0.09$; $df=1$; $P=1$).

3.3 *C. gestroi* alates and colonies

The richness values for colonies ($n=46$) ranged from 3 to 5 protist species, although most colonies (87%) harbored 4–5 species (Fig. 1a). Conversely, only 50% of alates ($n=24$;

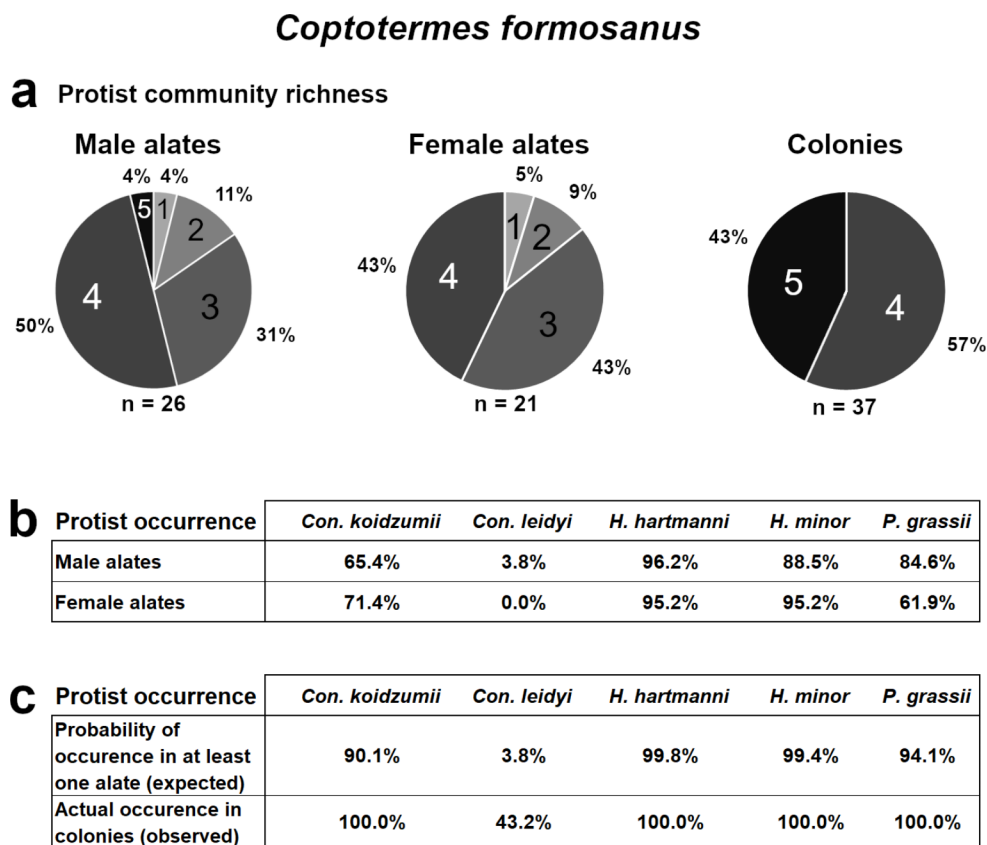
data from males and females combined) harbored 4–5 species (Fig. 1a). The values for alates (mean rank = 21.96) and colonies (mean rank = 42.57) significantly differed (Mann-Whitney $U=227$; $P<0.0001$). In line with this result, the richness distributions (two categories; 1–3 or 4–5 protist species) for alates and colonies also significantly differed (Fisher's exact test; $P=0.0014$). Compared to *C. gestroi* alates, a significantly greater proportion of *C. gestroi* colonies harbored 4–5 protist species.

3.4 *C. formosanus* alates and colonies

Similar to *C. gestroi*, all colonies ($n=37$) harbored 4–5 protist species (Fig. 2a). Contrastingly, only 49% of alates ($n=47$; data from males and females combined) harbored 4–5 species (Fig. 2a). Like *C. gestroi*, the richness values for alates (mean rank = 29.53) and colonies (mean rank = 58.97) significantly differed (Mann-Whitney $U=260$; $P<0.0001$). In agreement with this result, the richness distributions (two categories; 1–3 or 4–5 protist species) for alates and colonies also significantly differed (Fisher's exact test; $P<0.0001$). Compared to *C. formosanus* alates, a significantly greater proportion of *C. formosanus* colonies harbored 4–5 protist species.

Fig. 2 The results observed for *C. formosanus* alates and colonies.

(a) The numbers of protist species harbored by male alates, female alates, and colonies. (b) The percents of male and female alates that harbored each protist species. (c) The percent of colonies that harbored each protist species, and the probability of each species being harbored by at least one founding alate



3.5 *C. gestroi* protist occurrence

The percent of colonies ($n=46$) that harbored each protist species ranged from 56.5% for *H. bigfooti* to 100% for both *P. leei* and *Con. skunkapei* (Fig. 1c). For expected occurrence, the probabilities ranged from 55% for *H. bigfooti* to 100% for both *Cononympha* species (Fig. 1c). The expected and observed distributions for *H. batututi* were significantly different (MMI test; $X^2=8.12$; $df=1$; $P=0.0175$). The proportion of colonies that harbored this species was significantly greater than the expected proportion, i.e., *H. batututi* was harbored by more colonies than expected. In contrast, the other three chi-square tests performed for the MMI test indicated that there were no significant differences between the expected and observed distributions for *Con. monstrummogolloni* ($X^2=1.88$; $df=1$; $P=0.6794$), *H. bigfooti* ($X^2=0.04$; $df=1$; $P=1$), and *P. leei* ($X^2=0.17$; $df=1$; $P=1$).

3.6 *C. formosanus* protist occurrence

The percent of colonies ($n=37$) that harbored each protist species ranged from 43.2% for *Con. leidy* to 100% for the other four species (Fig. 2c). For expected occurrence, the probabilities ranged from 3.8% for *Con. leidy* to 99.8% for *H. hartmanni* (Fig. 2c). The expected and observed distributions for *Con. leidy* significantly differed

(MMI test; $X^2=17.18$; $df=1$; $P=0.0001$). The proportion of colonies that harbored this species was significantly greater than the expected proportion, i.e., *Con. leidy* was present in more colonies than expected. Contrastingly, the other two chi-square tests performed for the MMI test showed no significant differences between the expected and observed distributions for *Con. koidzumii* ($X^2=2.95$; $df=1$; $P=0.2580$) and *P. grassii* ($X^2=0.95$; $df=1$; $P=0.9874$).

4 Discussion

This study provides evidence for the importance of biparental symbiont transmission in protist-dependent termites. Our results indicate that the protist communities of conspecific male and female alates are similar with regard to species richness (Figs. 1a and 2a). Likewise, our results also show that conspecific male and female alates harbor their characteristic protist symbionts at similar frequencies (Figs. 1b and 2b). Similar to *Coptotermes* alates, the protist communities of male and female *Reticulitermes grassei* alates are alike with respect to both richness and species composition (Michaud et al. 2020). Our results also revealed that *Coptotermes* alates rarely harbor complete protist communities. Of the 71 alates sampled, only two harbored complete protist communities, whereas one protist species was absent

from 33 alates, and two or more species were absent from 36 alates (Supplementary Tables S3–S4). Likewise, *Reticulitermes* alates often do not harbor all of their characteristic protist species (Lewis and Forschler 2004; Michaud et al. 2020). Together, these results imply that male and female protist-dependent termite alates disperse harboring similarly rich protist communities that are often incomplete.

Our results also show that the protist communities of *Coptotermes* alates differ from those of colonies. Unlike alates, which frequently harbored 1–3 protist species, nearly all colonies harbored 4–5 species (Figs. 1a and 2a). Of the 83 colonies sampled, 39 harbored complete protist communities, while one protist species was absent from 38 colonies, and only six colonies were missing two species (Supplementary Tables S5–S6). The dissimilar richness values of alates and colonies imply that the initial workers of colonies acquire symbionts from both founding alates, i.e., via biparental transmission. This notion is supported by our results regarding expected and observed protist occurrence in colonies. For eight of the ten protist species, the proportions of colonies they were harbored by were either similar or identical to the expected proportions (Figs. 1c and 2c). These results imply that each protist species only needs to be present in one founding alate for colonies to acquire it. Because the protist communities of alates are frequently incomplete, it is likely that the communities of colonies are often the result of complementary contributions from both founding alates. Through biparental transmission, alate pairs that collectively harbor sufficiently rich protist communities that adequately digest wood are able to establish colonies despite the incompleteness of their individual communities.

Unlike most of the examined protists, the proportions of *C. gestroi* and *C. formosanus* colonies that harbored *H. batututi* and *Con. leidy*, respectively, were greater than the expected proportions (Figs. 1c and 2c). Considering the above findings, it is plausible that *H. batututi* and *Con. leidy* may actually be present in *C. gestroi* and *C. formosanus* alates, respectively, more often than our results indicate. In line with this notion, it is possible that these protist species were not molecularly detected in some alates because they were present in low abundances (Michaud et al. 2020). To assess the possibility that insufficient sequencing may have impacted our alate results, we performed a proportional-read-abundance analysis for each *Coptotermes* species (see materials and methods). For *C. gestroi* alates, the read depth of only one alate was below the computed threshold of 455 reads, which led us to conclude that insufficient sequencing likely did not impact our *C. gestroi* results and conclusions. For *C. formosanus* alates, the read depths of nine alates were below the calculated threshold of 667 reads, so, we performed the *C. formosanus* statistical analyses using the complete alate dataset and then repeated the analyses using

a reduced dataset that excluded these nine alates. Regarding statistical significance, the results from both datasets were identical, implying that insufficient sequencing also likely did not affect our *C. formosanus* results and conclusions.

Alternatively, it is also possible that, by chance, the *C. gestroi* and *C. formosanus* alates that were sampled harbored *H. batututi* and *Con. leidy*, respectively, less often than usual. While the sampled alates were collected from the same location as those used to establish colonies, not all alates were collected at the same time, i.e., during the same dispersal flight. This may have impacted our protist occurrence results, as the alates that comprised these two groups possibly flew from different colonies. However, this may also not be the case, as it is probable that we collected from the same *C. gestroi* and *C. formosanus* populations (i.e., the same pool of colonies) throughout our single-location sampling effort. Lastly, it is also conceivable that *C. gestroi* and *C. formosanus* colonies may be less likely to survive if *H. batututi* and *Con. leidy*, respectively, are absent. This may also explain the greater-than-expected proportions of colonies that harbored these protist species.

Our results imply that protist-dependent termite reproductives perform biparental transmission during colony establishment. This notion is supported by previous studies regarding *Reticulitermes* and *Coptotermes* reproductives (Shimada et al. 2013; Brossette et al. 2019; Michaud et al. 2020; Velenovsky et al. 2021). Biparental transmission has only been found to potentially occur in a handful of organisms, including leafhoppers (Watanabe et al. 2014), earthworms (Paz et al. 2017), aphids (Moran and Dunbar 2006), mosquitos (Damiani et al. 2008), and tsetse flies (De Vooght et al. 2015). For most of these hosts, the symbionts that may be biparentally transmitted are facultative bacterial species, which are usually sufficiently acquired by offspring through maternal symbiont transmission (Moran and Dunbar 2006; Damiani et al. 2008; Watanabe et al. 2014; De Vooght et al. 2015). In contrast, offspring of the lumbricid earthworm, *A. tuberculata*, probably often need both maternal and paternal lineages of *Verminephrobacter* to obtain sufficient inoculums (Paz et al. 2017). Unlike *Verminephrobacter*, which may be a facultative symbiont (Lund et al. 2010; Viana et al. 2018), the microbes that protist-dependent termites likely biparentally transmit are obligate symbionts (Cleveland 1923, 1924). Like lumbricid earthworms, biparental transmission is probably often necessary for protist-dependent termite colonies to acquire sufficient symbiont communities, as alates harbor small inoculums that are frequently incomplete during dispersal (Lewis and Forschler 2004; Shimada et al. 2013; Michaud et al. 2020; Velenovsky et al. 2021). In short, both lumbricid earthworms and protist-dependent termites are unique among hosts because their

offspring likely often require biparental transmission to obtain adequate inoculums (Paz et al. 2017).

Protist-dependent termites also differ from other hosts of biparentally transmitted symbionts in additional aspects. In most of the aforementioned hosts, paternal symbiont lineages are initially acquired by females through mating before they are transmitted to offspring, i.e., males do not directly transmit symbionts to progeny (Moran and Dunbar 2006; Damiani et al. 2008; De Vooght et al. 2015). Likewise, hermaphroditic lumbricid earthworms acquire symbionts during mating that are subsequently transmitted to offspring (Paz et al. 2017). Unlike these hosts, progeny of the leafhopper *Nephotettix cincticeps* can directly acquire paternal symbiont lineages through intrasperm transmission (Watanabe et al. 2014). Protist-dependent termite kings likely also directly transmit their symbionts, as both queens and kings donate proctodeal fluids to their initial workers during colony foundation (Shellman-Reeve 1990; Brossette et al. 2019; Velenovsky et al. 2021). Therefore, unlike the above symbioses, biparental transmission in protist-dependent termites does not involve mating, but instead occurs through post-embryonic offspring consuming hindgut fluid donations (Nalepa 2011), highlighting once again the importance of gut fluid exchanges in social insects (LeBoeuf et al. 2016). While kings and queens may exclusively transmit their own symbionts, it is also possible that each reproductive transmits both maternal and paternal symbiont lineages, as kings and queens also provide proctodeal fluids to one another during colony establishment (Shellman-Reeve 1990; Rosengaus and Traniello 1991; Brossette et al. 2019). These donations could cause the symbiont communities of reproductives to homogenize before their initial workers emerge. It remains to be determined if queens and kings transmit symbionts to each other during colony foundation.

In agreement with our results regarding *Coptotermes* colonies, *Reticulitermes* (Kitade and Matsumoto 1993; Michaud et al. 2020), *Hodotermopsis* (Kitade et al. 2012), *Zootermopsis* (Taerum et al. 2018), and *Heterotermes* (Jasso-Selles et al. 2017; De Martini et al. 2021) colonies also sometimes harbor incomplete protist communities. Together, these results suggest that colonies can potentially survive even if they are established by alate pairs with incomplete communities. The constitutions of termite protist communities possibly allow colonies that harbor incomplete communities to still be successful, as multiple protists may produce lignocellulolytic enzymes with similar functions. Whether this hypothesis has substance is unclear, as the functional roles of individual protist species within communities have not been investigated, except in *C. formosanus*. Both Lai et al. (1983) and Yoshimura (1995) observed that *C. formosanus* protists are differentially distributed within the hindgut, suggesting that each protist may have a specific

role in wood digestion. In line with this notion, Nishimura et al. (2020) found that each of the protists harbored by *C. formosanus* expresses a distinct profile of lignocellulolytic enzymes. However, the digestive role of each protist may be somewhat alike, as some of the enzymes have similar functions (Nishimura et al. 2020). In short, the roles of individual protist species within termite communities are almost entirely undetermined, making it unclear if the success of colonies with incomplete communities is at all attributable to functional redundancy, i.e., protist species compensating for the absence of other symbionts.

Our observations confirm that protist-dependent termite alates have not evolved a mechanism that ensures they disperse with complete symbiont communities. Why such a mechanism has not been selected for may be explained by the dispersal strategies of termites, which involve hundreds or thousands of simultaneously flying alates (Weesner 1960, 1970; Nutting 1969; Chouvenc et al. 2017). Most alates (>99%) that disperse actually fail to establish new colonies, primarily due to either predation or adverse meteorological conditions during flight or courtship behaviors (Nutting 1969; Chouvenc et al. 2017; Chouvenc 2019). Alates fortunate to form pairs and produce copulariums may also fail to establish colonies if they succumb to bacterial or fungal pathogens (Rosengaus and Traniello 1993; Rosengaus et al. 2011). Furthermore, alates may fail to establish colonies if their morphological and physiological characteristics are not conducive to successful foundation (Shellman-Reeve 1996; Mullins and Su 2018; Chouvenc 2019; Inagaki et al. 2020).

This study reveals an important aspect of protist-dependent termite biology. Because only a minute fraction of the alates that disperse establish colonies, it is inconsequential if most leave their natal colonies harboring incomplete symbiont communities (Chouvenc 2022). As long as a sufficient number of alates are involved in a dispersal event, there will be a few lucky alate pairs that survive dispersal, harbor complementary, adequate protist communities, and establish colonies. Compared to predation and adverse weather (Nutting 1969), alates harboring incomplete protist communities in all likelihood only marginally affects colony foundation success. Although vertical transmission in protist-dependent termites is imperfect, it is also adequate, as biparental transmission allows founding kings and queens to jointly transmit sufficiently rich symbiont communities to their initial workers.

In conclusion, our study provides evidence that protist-dependent termite reproductives perform biparental symbiont transmission during colony establishment. Biparental transmission of cellulolytic protists probably initially arose in the subsocial woodroach-like ancestor of termites and *Cryptocercus* (Nalepa 1984, 2015), and this mechanism

may be conserved in Termitidae, even though protists were lost in this family (Chouvenc et al. 2021; Sinotte et al. 2022). Here, we argue that biparental colony foundation has allowed termite-protist symbioses to be maintained despite imperfect vertical transmission. While beneficial in this regard, biparental transmission may also be problematic, as there is the potential for conflict to occur among symbiont lineages of different parental origins. In a future study, we will investigate this and other aspects of symbiosis in protist-dependent termites using hybrid *Coptotermes* colonies established by *C. gestroi* and *C. formosanus* alates (Chouvenc et al. 2015).

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Declarations

Competing interests The authors declare that they have no conflict of interest.

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