Fungal Ecology 26 (2017) 20–27

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Methodological Advances

Sex and diversity: The mutualistic and parasitic fungi of a fungus-growing termite differ in genetic diversity and reproductive strategy

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ARTICLE INFO

Article history:
Received 23 January 2016
Received in revised form
17 October 2016
Accepted 8 November 2016

Corresponding Editor: Duur Aanen

Keywords:
Fungus-growing termite
Odontotermes obesus
Termitomyces
Pseudoxylaria
Parasite
Mutualism
Sexual reproduction
Clonality
Diversity
OTU

ABSTRACT

Sex in symbionts is useful in creating beneficial traits. However, the resultant decrease in genetic relatedness between symbionts can be potentially detrimental for mutualistic interactions such as those between fungus-growing termites and the cultivated fungi in the genus *Termitomyces*, where the termite host should try to increase intra-nest symbiont relatedness to increase crop productivity. Any parasites of this mutualism such as the fungi in the sub-genus *Pseudoxylaria* may also use sex to generate variation to counter evolving host defensive mechanisms. Using molecular phylogenetic tools, we found within-nest genetic homogeneity in *Termitomyces* species but not in *Pseudoxylaria* species associated with the fungus-growing termite *Odontotermes obesus*. There was lower OTU but higher genotypic diversity (within the most abundant OTU) in the genus *Termitomyces* than in the sub-genus *Pseudoxylaria*. Additionally, population genetics methods suggest a sexual population structure for *Termitomyces* species and clonal propagation for *Pseudoxylaria* species. This is the first study to investigate the population genetics of the symbiotic fungi associated with the genus *Odontotermes* from India.

1. Introduction

Sex is a reproductive strategy that can create and facilitate the spread of beneficial traits including parasite resistance, and can also purge deleterious mutations (Hurst and Peck, 1996). However, sexual reproduction may also give rise to conflict between organisms such as host and symbiont which are in a mutualistic interaction, as it may lead to the emergence of symbiont variants which may compete with each other and reduce host fitness (Frank, 1996). Consequently, it is generally believed that clonal propagation of symbionts within hosts will be beneficial for host fitness (Frank, 1996; Korb and Aanen, 2003). Parasites, on the other hand, need to evolve counter mechanisms against host anti-parasite mechanisms to win the 'arms race' and thus may use sex as a strategy to generate required variation (McDonald and Linde, 2002). However, once adapted to the host, parasites may multiply clonally thus limiting sexual reproduction (Heitman, 2006). Therefore, organisms could differ in the reproductive strategies that they demonstrate depending on their position and adaptive status in the multipartite network of interactions with other organisms.

In the tripartite interaction between fungus-growing termites (subfamily: Macrotermitinae) and two fungi—genus *Termitomyces* (Basidiomycota) and sub-genus *Pseudoxylaria* (Ascomycota)—the first two are partners engaged in an obligate mutualism whereas the third is a parasite (Batra and Batra, 1979; Visser et al., 2011). Inside the termite nest, the mutualistic genus *Termitomyces* is cultivated on partially digested plant matter called a fungus comb. Apart from growing as mycelia, it also produces asexual spores in structures called mycotêtes which, when ingested by termites and deposited on fresh substrate with termite droppings, act as new sources of inoculum, resulting in clonal propagation of *Termitomyces* species inside the nest (Leuthold et al., 1989; Aanen, 2006).

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http://dx.doi.org/10.1016/j.funeco.2016.11.003
1754-5048/

Between nests, the mutualistic fungus is vertically (and clonally) or horizontally (and sexually) transmitted (Korb and Aanen, 2003). In the horizontal mode (Korb and Aanen, 2003), genetically different spores brought into the nest can germinate into mycelia (homokaryons) which may fuse to form a heterokaryon (Korb and Aanen, 2003; De Fine Licht et al., 2005; Nobre et al., 2014). Heterokaryons may produce sexual fruit bodies (mushrooms), via a heterothallic mating system (De Fine Licht et al., 2005), which fruit outside the nest, dispersing spores which can be acquired by worker termites of incipient colonies. Due to this horizontal nature of fungal acquisition, multiple fungal genotypes can be present inside the nest, which may result in competition between different fungal genotypes for host resources, thus reducing overall productivity (Aanen, 2006; Aanen et al., 2009). Therefore, termites employ mechanisms to increase genetic relatedness of the mutualistic fungus inside the nest as elucidated for *Macrotermes natalensis* (Aanen et al., 2009). The resultant decrease in competition between fungal variants possibly leads to long-term association of one fungal genotype per termite colony (Aanen et al., 2009). The presence of the third interactant, the parasitic sub-genus *Pseudoxylaria*, is not evident at first inside the nest but it starts growing vigorously on the fungus combs that are left untended by termites (Batra and Batra, 1979; Hsieh et al., 2010). Since *Pseudoxylaria* species are not evident on healthy fungus gardens but appear only in deteriorating gardens, they appear to practice a sit-and-wait strategy and have been called stowaway parasites (Visser et al., 2011). However, it is unclear from where *Pseudoxylaria* species are acquired. Although *Pseudoxylaria* species do not seem to have coevolved as parasites in this system, they show specificity to fungus-growing termites, since all *Pseudoxylaria* species isolated from termite nests cluster in a separate termite-associated clade (Visser et al., 2009; Hsieh et al., 2010). However, unlike the mutualistic genus *Termitomyces*, no definite information is available on the mode of reproduction and transmission of the sub-genus *Pseudoxylaria*. Therefore, in this tripartite interaction, the mutualistic fungus reproduces clonally inside the termite nest and (mostly) sexually outside the nest but the mode of reproduction of the parasitic fungus is elusive.

For fungi, demonstrating sexual reproduction in the laboratory may be feasible, but this only reflects their potential to undergo sexual reproduction; whether they actually reproduce sexually in the wild can only be evaluated using population genetic methods (Tibayrenc et al., 1991) as has been done for *Termitomyces* species associated with the termite genus *Macrotermes*. In *Termitomyces* species associated with *M. natalensis*, under horizontal transmission and frequent recombination, alleles were in Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (De Fine Licht et al., 2006). On the other hand, under vertical transmission and clonal reproduction, deviations from HWE and significant linkage disequilibrium (LD) across loci were expected (De Fine Licht et al., 2006). For the termite genus *Odontotermes*, a horizontal mode of transmission has been suggested for the mutualistic fungus (Aanen et al., 2002, 2007; Katoh et al., 2002) and mushrooms were observed in nests of two species (*Odontotermes aff. pauperans* and *Odontotermes* sp. 2) (Koné et al., 2011). Osiemo et al. (2010) also attributed the high diversity of fungal symbionts associated with the termites that they studied, including the genus *Odontotermes*, to horizontal transmission. However, sexual reproduction (as a result of horizontal transmission) of *Termitomyces* species associated with the genus *Odontotermes* has not been confirmed decisively, e.g. using extensive population genetics methods. Therefore, the objectives of this present study were to investigate the genetic diversity and population genetic structure of *Termitomyces* and *Pseudoxylaria* strains associated with the termite *Odontotermes obesus* since there is no information on these fungi associated with *O. obesus* or from any *Odontotermes* in the Indian subcontinent.

2. Materials and methods

2.1. Sampling

Termitomyces and *Pseudoxylaria* samples were isolated from fungus combs collected from 29 *O. obesus* nests present in three different localities of Bangalore (India) (Fig. 1 and Table S1). *O. obesus* is a widely distributed species in India except the colder regions, and especially prevalent in South India (Bose, 1984; Chhotani, 1997). Special care was taken while sampling the fungus combs to keep the sampling as sterile as possible.

2.2. Fungal culture for DNA extraction

Individual mycotêtes were picked from freshly collected fungus combs and inoculated on potato dextrose agar (PDA), and incubated at 30 °C for 8–12 d. The growing mycelium of *Termitomyces* was subcultured on PDA and the pure culture was used for DNA isolation. *Pseudoxylaria* was isolated from stromata developing on fungus combs kept at room temperature in autoclaved plastic bags. A small piece of stromata was inoculated onto PDA and incubated at 30 °C for 2–3 d. Growing mycelia showing *Pseudoxylaria*-like morphology were subcultured on PDA and the pure culture was used for subculturing in potato dextrose broth (PDB) for DNA isolation. DNA was extracted from subcultured fungi by the Lee and Taylor (1990) method.

2.3. PCR and sequencing

The entire *ITS1–5.8S–ITS2* (450–700 bp) region was amplified and sequenced using the primers ITS1 (5′–TCCGTAGGTGAACCTGCGG) and ITS4 (5′–TCCTCCGCTTATTGATATGC) (White et al., 1990). PCR amplifications were done in a reaction mixture (25 μL or 50 μL) of the following composition: dNTP mix (0.2 mM), primers (2 μM), BufferA (Bangalore Genei, 1X) and *Taq* polymerase (Bangalore Genei, 0.2 or 0.4 μL). The following thermal cycles were used in a MJ Research PTC-150 MiniCycler™: initial denaturation at 95 °C (3 min) followed by 30 cycles of denaturation (95 °C, 30 s), annealing (60 °C, 30 s), extension (72 °C, 1 min) and a final extension at 72 °C (5 min). Amplified products were purified by QIAquick Gel Extraction Kit and sequenced by Eurofins Genomics India and the DNA sequencing facility at the National Centre for Biological Sciences (NCBS), Bangalore. Two samples per nest were used for both fungi except for two nests where three samples were analyzed instead. All sequences were deposited into GenBank (National Center for Biotechnology Information) under the accession numbers KC848186–KC848216 (*Termitomyces*), KX646673–KX646700 (*Termitomyces*), KC848217–KC848247 (*Pseudoxylaria*) and KX646651–KX646672 (*Pseudoxylaria*).

2.4. Data analysis

All the nucleotide sequences thus obtained were analyzed using BLAST. *Termitomyces* sequences showing double peaks were coded using standard IUPAC codes, but if the double peak represented a base or a gap (indel), the coding employed alphabets O (T/gap) and Q (C/gap) (Aanen et al., 2009). Sequences were aligned in MAFFT online version 7 (Katoh and Standley, 2013) using L-INS-I (gap opening penalty = 1.5, gap extension penalty = 0.14; 1PAM/κ = 2 scoring matrix for nucleotide sequences) (Nobre et al., 2011b). Overhangs of aligned sequences having missing data were removed in MEGA version 6.06 (Tamura et al., 2013). Neighbor-joining (NJ) phylograms were constructed in PAUP (Swofford, 2002) using the distance matrix (uncorrected pairwise differences) generated in

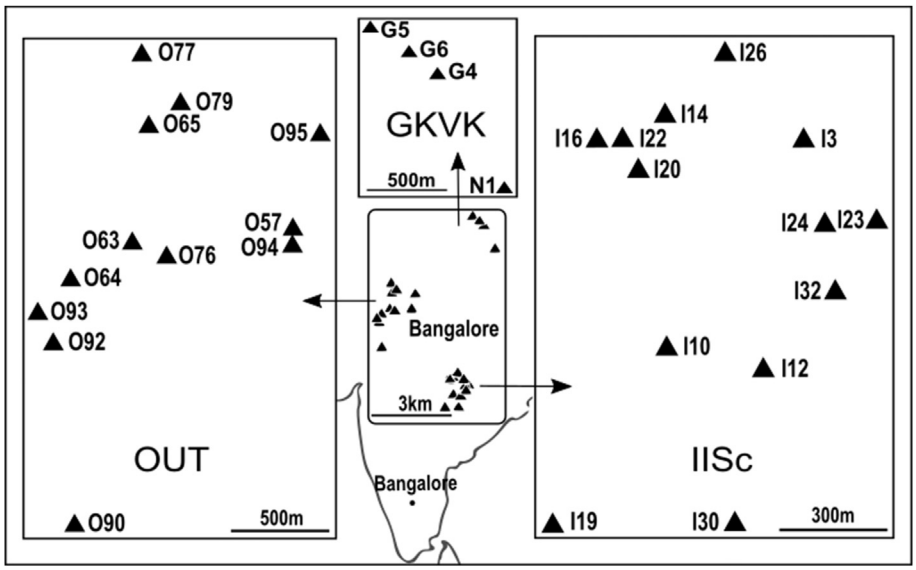

Fig. 1. Map of Bangalore (India) showing positions of termite nests sampled in this study from three different locations labelled as OUT (12 nests), Gandhi Krishi Vignan Kendra (GKVK; 4 nests) and Indian Institute of Science (IISc; 13 nests).

Mothur version 3 (Schloss et al., 2009), with each gap treated as a single position. Trees were visualized in Dendroscope v3.5.1 (Huson and Scornavacca, 2012) and edited in Inkscape v0.91 (www.inkscape.org). Sequences sharing 98% similarity were clustered in operational taxonomic units (OTUs) in Mothur version 3 (Schloss et al., 2009) using alignment as the input. A representative sequence from each OTU was analyzed using BLAST (Tables S2A and S2B).

For population genetics analysis, sequences belonging to only the most abundant OTU of *Termitomyces* as well as *Pseudoxylaria* were used. One sample per nest was used for both fungi if intra-nest sequences were identical; otherwise both sequences were used (*Termitomyces*, N = 26; *Pseudoxylaria*, N = 28) in the aligned format (Table 1) such that all sequences belonged to the same OTU. Double peaks in *Termitomyces* sequence chromatograms were coded as single nucleotide polymorphisms (SNPs) (De Fine Licht et al., 2006; Nobre et al., 2011a). *Termitomyces* sequence data (18 SNPs only) were first converted into a genalex-formatted file using GenAlEx 6.502 (Peakall and Smouse, 2006, 2012) and then edited manually to convert them into diploid data before importing them into Rstudio 0.99.491 (RStudio Team, 2015) user interface for R 3.2.3 (R Core Team, 2015) for analysis. The SNPs (*Termitomyces*) and polymorphic sites (*Pseudoxylaria*) within the *ITS* region were treated as single loci and were therefore used in a multilocus genotype (MLG) framework for all analyses. *Termitomyces* and *Pseudoxylaria* sites were treated as codominant and haploid respectively. Unique genotypes, Simpson's index and evenness were obtained with the R-package 'poppr' 2.1.0 (Kamvar et al., 2014, 2015). Samples from the three locations (Fig. 1) labelled as OUT (12 nests), GKVK (4 nests) and IISc (13 nests) were treated as sub-populations of a single population. To examine population structuring at the subpopulation level that may lead to the Wahlund effect, analysis of molecular variance (AMOVA) calculations were done in the R-package 'poppr' 2.1.0 (Kamvar et al., 2014, 2015) with subpopulation as the hierarchy level tested. AMOVA calculations were repeated using the clone-correction option to account for the high degree of clonality. Φ fixation index (among subpopulations) was calculated and the significance of the fixation index was tested using 1000 permutations.

For *Termitomyces*, the SNPs were tested for deviations from Hardy-Weinberg equilibrium (HWE) using the exact test based on Monte Carlo permutations of alleles with 1000 replicates in the R-package 'pegas' (Paradis, 2010). No such test was possible for the haploid *Pseudoxylaria*. Association between polymorphic loci was tested using the standardised index of association (rbarD) test available in the R-package 'poppr' 2.1.0 (Kamvar et al., 2014, 2015). To evaluate the significance of rbarD, alleles were randomly permuted 1000 times among individuals. rbarD calculations were also done with clone-corrected data. Parsimony tree length permutation tests (PTLPT) were performed in PAUP (Swofford, 2002) with parsimony as the optimality criterion with the following settings: heuristic search with random addition sequences; 1000 replicates; tree bisection–reconnection (TBR) branch swapping. To test whether recombination was completely free, the length of the actual tree was compared to the length of 1000 randomized data sets using the 'permute' option in PAUP (Swofford, 2002) with the

Table 1
Characteristics of the *ITS* locus of *Termitomyces* species (OTU1) and *Pseudoxylaria* species (OTU1) isolated from *O. obesus* nests.

Fungus	N_T	N	L	SNPs/Ps	HWE	G	G:N	λ	E	rbarD	p^r	PTLPT	Clone corrected		
													rbarD	p^r	PTLPT
Termitomyces (OTU1)	48	26	585	18	56	14	0.54	0.88	0.72	0.21	0.001	0.001	0.14	0.001	0.452
Pseudoxylaria (OTU1)	42	28	424	12	–	7	0.25	0.38	0.41	0.37	0.001	–	0.27	0.001	–

N_T – total number of samples belonging to OTU1 sequenced from all the nests; N – sum of number of unique samples from each nest belonging to OTU1; L – aligned sequence length; SNPs – number of SNPs for *Termitomyces* species; Ps – number of polymorphic sites for *Pseudoxylaria* species; HWE – percentage of polymorphic sites that did not depart significantly from HWE; G – number of unique *ITS* genotypes; G:N – ratio of the number of unique *ITS* genotypes found over the sample size; λ – Simpson's index; E – evenness; rbarD – standardized index of association; p^r – p-value associated with rbarD; PTLPT – significance level of parsimony tree length permutation test (PTLPT) based on comparison of length of observed parsimony tree to lengths of trees generated from 1000 permuted data sets; – – Test not possible.

following settings: 1000 replicates; heuristic search with random addition sequences (nreps = 10); tree bisection–reconnection (TBR) branch swapping. For a freely recombining population, actual tree length will be similar to tree lengths generated from a randomised data set, whereas for a clonal population, actual tree length will be much shorter. PTLPT analyses were also performed on clone-corrected data.

All figures were processed in Inkscape v0.91 (www.inkscape.org) and GIMP v2.8.14 (www.gimp.org).

3. Results and discussion

3.1. Genetic diversity

3.1.1. Termitomyces

Samples of *Termitomyces* isolated from the same nest were always identical except for three nests ('I32' (raw sequences), 'O65' and 'O93') (Fig. 2A). Even for these nests, the difference was only in the karyotic status of the two sampled isolates, i.e. one isolate was the homokaryon of the heterokaryotic isolate; therefore, even these were 'identical', confirming results from other studies (Katoh et al., 2002; Shinzato et al., 2005; Aanen et al., 2009) that *Termitomyces* occurs as a monoculture within nests. However, this is the first study which has found both the homokaryon and heterokaryon from the same nest, unlike earlier studies which isolated only a heterokaryon (Aanen et al., 2009; Nobre et al., 2014). This monoculture can be established by high spore inoculation density and fusion between closely related mycelia possibly resulting in lifetime partner fidelity between a *Termitomyces* genotype and a termite colony (Aanen et al., 2009). In two nests ('I12' and 'G4'), we found identical *Termitomyces* sequences when nests were resampled after 2–5.5 months, which suggests long-term association between termites and a particular cultivar, a finding not confirmed by earlier studies. All *Termitomyces* sequences clustered in 2 OTUs with most of the samples belonging to a single OTU (OTU1 had 48 out of the total 59 sequences) (Fig. 3A). In other words, out of the 29 nests sampled, 24 harboured the same OTU while the other 5 had OTU2. The high prevalence of *Termitomyces* OTU1 may indicate its status as a 'better crop variety' and or its high colonisation ability. Only those strains of *Termitomyces* species that produce more mycotêtes than others (hence 'better crop varieties') can survive inside the nest (Aanen, 2006; Aanen et al., 2009). In *Termitomyces* OTU1, there were 14 unique *ITS* genotypes out of 26 isolates sampled (Table 1, Fig. 3A). There was high genotypic diversity (λ) and evenness (E) (Table 1) indicating many different and similarly abundant unique genotypes. At this local scale of study (~23 km^2) we also found that the same genotypes can be shared by different nests (Fig. 2A). However, this does not mean that two nests situated close to each other will have the same genotype, e.g. nests 'G5' and 'G6' are within a few km of each other (Fig. 1) but they do not share identical genotypes (Fig. 2A). Also, nest 'G4' which is situated near 'G5' and 'G6' harbours a different OTU (OTU2). This may be because of temporal variation in the availability of different genotypes, in the form of spores derived from mushrooms at nest inoculation, in the same locality. Although mushrooms have been observed in several nests, they are difficult to track owing to their limited longevity and harvesting by humans for consumption.

3.1.2. Pseudoxylaria

We could isolate *Pseudoxylaria* with a high success rate of ~93%; only two nests 'I32' and 'O76' did not yield *Pseudoxylaria* (Table S1) which could be because of insufficient sampling. This shows the high prevalence of *Pseudoxylaria* species in *O. obesus* nests. Visser et al. (2009) also found the highest prevalence of *Pseudoxylaria* species (~83.3%) in combs of the genus *Odontotermes* as compared to those of genera *Macrotermes* and *Microtermes*. This may indicate

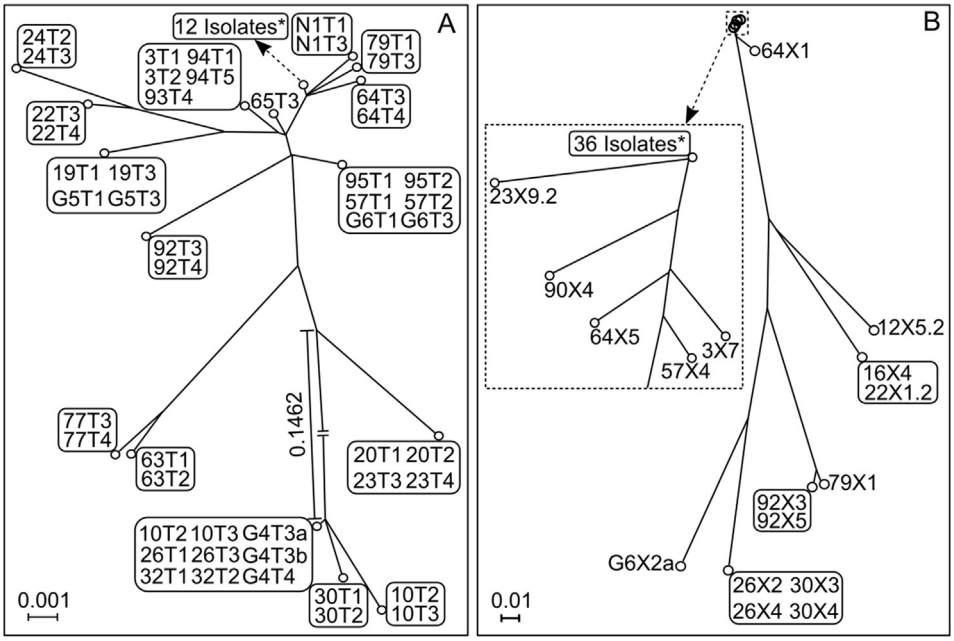

Fig. 2. Unrooted phylograms showing genetic diversity of heterokaryotic genus *Termitomyces* (A) (59 samples; sequence length: 585 bp) and haploid subgenus *Pseudoxylaria* (B) (53 samples; sequence length: 424 bp) isolated from nests of the fungus-growing termite *O. obesus*. Uncorrected pairwise differences i.e., p-distance (but including indels) were used for making neighbour-joining phylograms. In the labels, numerals after T (*Termitomyces*) or X (*Pseudoxylaria*) represent the sample number collected from the nest and the numeral and/alphabet before T or X indicates the nest identity. The dashed insert in (B) shows a magnified view of the small dashed box as indicated by the arrow. Identical isolates are clubbed together in rounded-edged rectangular boxes. '12 Isolates*' and '36 Isolates*' in (A) and (B) respectively represent 12 and 36 identical *Termitomyces* and *Pseudoxylaria* isolates other than those shown here (details of these isolates available in Table S1). Unfilled circles indicate nodes. Actual branch lengths are indicated wherever branches are too long to represent. The scales indicate uncorrected pairwise difference.

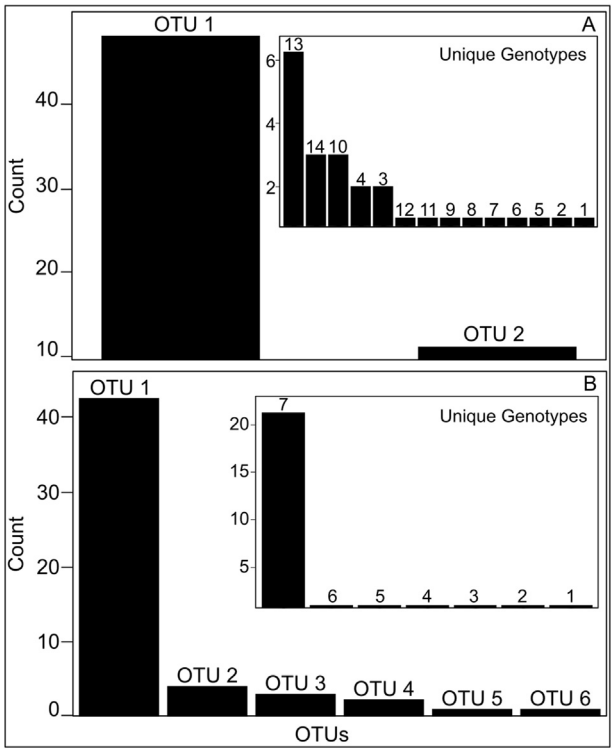

Fig. 3. OTU diversity and genotypic evenness: distribution of OTU and genotype abundances. Frequency distribution of all OTUs for both the fungi, mutualistic *Termitomyces* species (A) and parasitic *Pseudoxylaria* species (B) isolated from nests of the fungus-growing termite *O. obesus*. OTU labels are written above the bars. Inserts show frequency distribution of unique *ITS* genotypes of OTU1. Genotype labels are written above the bars without the suffix 'G'. Height of each bar represents frequency of that particular OTU/genotype.

that *Odontotermes* nests are particularly susceptible to *Pseudoxylaria* invasion. All *Pseudoxylaria* sequences clustered in 6 OTUs with most of the samples belonging to a single OTU (OTU1 had 42 out of the total 53 sequences) (Fig. 3B). Unlike *Termitomyces* where each nest had the same genotype, multiple OTUs of *Pseudoxylaria* occurred within the same nest confirming earlier studies (Visser et al., 2009) (Fig. 2B) when sampled at different times (1–8 months apart), e.g. 12X2.2 (OTU1) and 12X5.2 (OTU5), 22X1.2 (OTU4) and 22X4 (OTU1) etc. (Table S1). Also, out of the 29 nests sampled, OTU1 was found in 24 nests whereas the other 5 were found in only 1–2 nests. The high prevalence of *Pseudoxylaria* OTU1 could be because of its high success rate in colonising termite nests. Within the most abundant OTU1, there was low genotypic diversity (λ) and evenness (E) (Table 1) indicating that only a few genotypes were present, with the population being dominated by only a few of them. In total, there were only 7 unique *ITS* genotypes out of 28 isolates sampled (Table 1), G7 being the most abundant genotype (Fig. 3B) occurring in 22 different nests (located as far apart as ~7.5 km). Also, this genotype could be re-isolated from the same nest when sampled at a different time (24X1.2 and 24X5 (both G7)).

3.2. Population genetic structure: sex or clonality?

3.2.1. Termitomyces OTU1

The *ITS* region of 585 bp length revealed a total of 18 SNPs which were used for all the analyses. The 14 unique genotypes out of 26 samples belonging to OTU1 resulted in a G:N < 1 (Table 1) indicating overrepresentation of some genotypes. Only one genotype was present at a higher frequency than others and many were recorded only once (Fig. 3A). The AMOVA results revealed no sign of population structuring whether the analysis was done with all the individuals or using only clone-corrected subpopulations (i.e. only one unique clone being retained in each subpopulation to remove any artefact resulting from overrepresented clones) ($p > 0.05$) (Table S3; Figs. S1A and B). Variation at this level accounted for only ~4% of the total variation (Table S3). Therefore, the rest of the analysis was performed at the population level, i.e. all 26 sequences were used.

The high genetic diversity (λ) and evenness seemed to be indicative of sexual reproduction. More than half of the SNPs were in HWE (Table 1) suggesting the presence of sexual reproduction. However, the test for association between loci (rbarD), which is based on analysis of genetic distances among loci and also accounts for the number of loci sampled, was significant ($p = 0.001$) rejecting the null hypothesis of no linkage between loci whether the analysis was done with all the individuals or with clone-corrected data (Table 1; Fig. 4A and B). However, the value of clone-corrected rbarD (standardised index of association) decreased to ~1.5 times that of the original (Table 1). The PTLPT analysis showed that the length of the parsimony tree (actual length) is significantly shorter than the length of the trees obtained from a randomized data set (a freely recombining population) ($p = 0.001$) (Table 1, Fig. 5A). However, when this analysis was done with clone-corrected data, the actual length was not significantly different from the length of the trees representing a freely recombining population ($p = 0.452$) (Table 1, Fig. 5B). These results together indicate that recombination is occurring in the population albeit at a low level leaving its signature in the population which was unmasked by clone-correction. Therefore, *Termitomyces* species associated with *O. obesus* appear to have a sexual population structure with signatures of recombination. These results are similar to other studies on *Termitomyces* species associated with *Macrotermes* termites (De Fine Licht et al., 2006; Nobre et al., 2011a). De Fine Licht et al. (2006) found all sites in the *ITS* locus to be in HWE and most in linkage equilibrium for a sexually reproducing *Termitomyces* population. Additionally, Nobre et al. (2011a) found all sites in the *ITS* locus to be in HWE and most in linkage equilibrium for a sexually reproducing population. However, these results were based on only 6 SNPs. Using another marker, i.e. *EF1α*, which had many more polymorphic sites, Nobre et al. (2011a) found most of the sites in HWE for sexually reproducing *Termitomyces* as compared to clonally reproducing *Termitomyces* species which had very few sites in HWE. In contrast, they found fewer sites in linkage equilibrium for clonally reproducing *Termitomyces* as compared to sexually reproducing *Termitomyces* species. Also, for the genus *Odontotermes* which is widely distributed in Africa and Asia, a horizontal mode of fungal transmission and thereby sexual reproduction has been suggested but not confirmed (Aanen et al., 2002, 2007; Katoh et al., 2002). Our results lend support to such a possibility. Even though our results are similar to other studies, the difference in the level of sexual recombination could be due to a difference in the geographical scale of sampling; Nobre et al. (2011a) studied the population structure of *Termitomyces* at a much larger geographical scale than our study and, therefore, probably captured more variation. Nonetheless, it will be interesting to do a comparative investigation of *Termitomyces* associated with other *Odontotermes* species to determine if termites govern the mode of reproduction of their cultivar, as shown for the genus *Macrotermes*, where some associated *Termitomyces* species show a population structure indicative of sexual reproduction while others demonstrate a clonal population structure (Nobre et al., 2011a).

3.2.2. Pseudoxylaria OTU1

Most ascomycetes spend a major part of their life cycle in the haploid state (Burt et al., 1996; Nelson, 1996). In our study, there

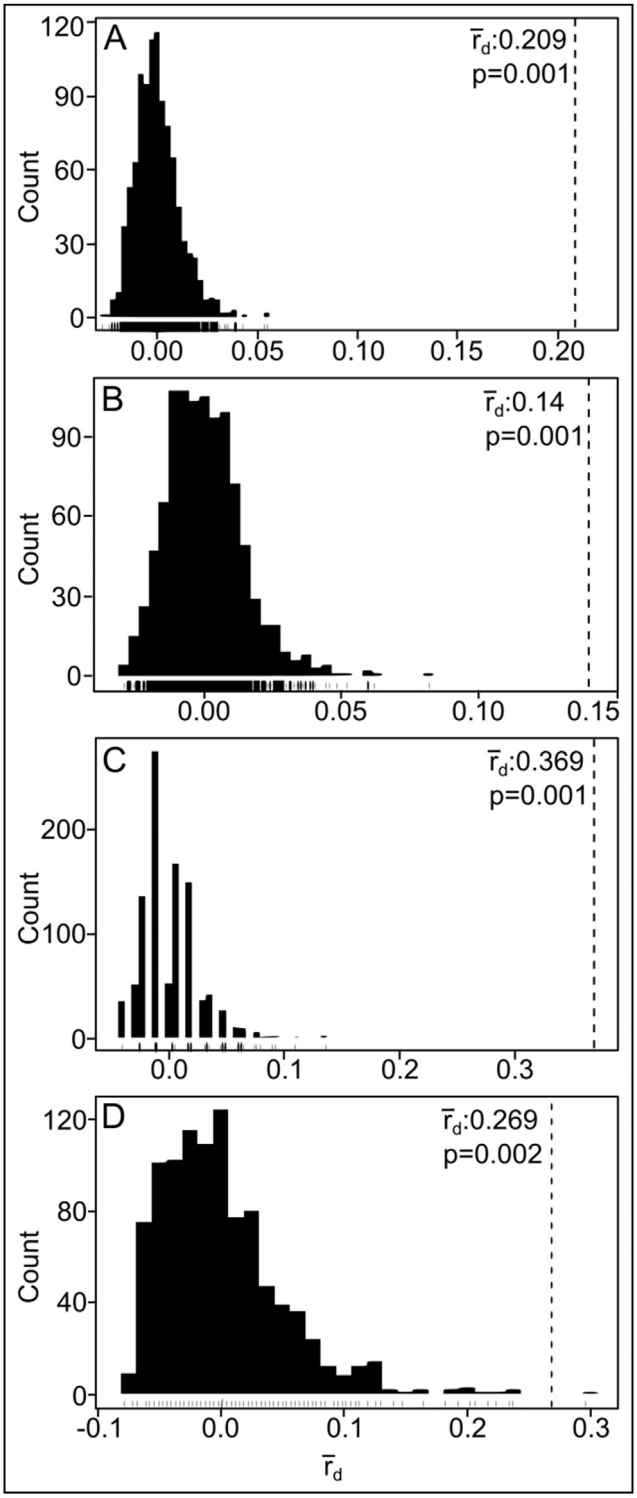

Fig. 4. Standardized index of association. Frequency distribution (FD) of standardized index of association (rbarD) for randomized data of the two fungi, mutualistic *Termitomyces* species (OTU1) and parasitic *Pseudoxylaria* species (OTU1) (distribution expected under no linkage). Observed rbarD for the actual data is shown with a dashed line. FD for analysis with all individuals of *Termitomyces* species (A) and clone-corrected data (B). FD for analysis with all individuals of *Pseudoxylaria* species (C) and clone-corrected data (D).

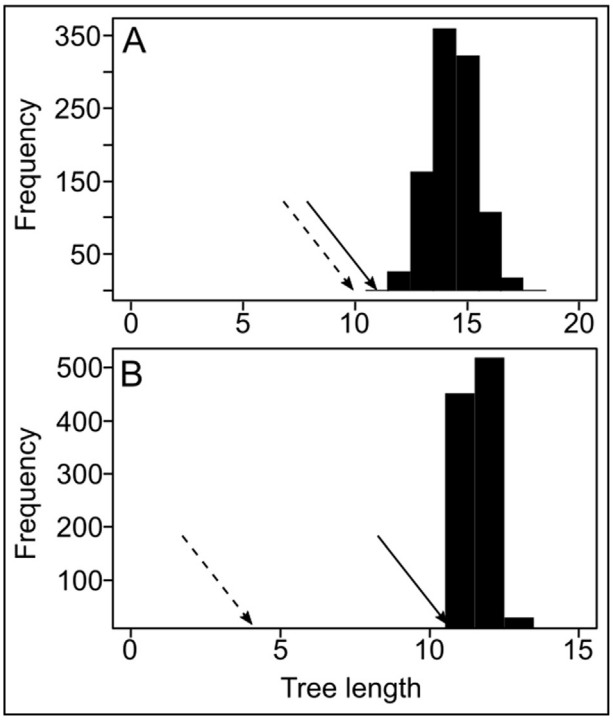

Fig. 5. Parsimony tree length permutation test (PTLPT) analysis. Frequency distribution (FD) of parsimony tree lengths for randomized data (distribution expected under no linkage) of the mutualistic fungus *Termitomyces* species (OTU1) polymorphic sites. Positions of the actual length of the tree and the minimum length possible are marked with solid and dashed arrows respectively. FD for analysis with all individuals of *Termitomyces* species (A) and clone-corrected data (B).

was a complete absence of double peaks in the DNA chromatograms for *Pseudoxylaria* suggesting a completely haploid state within the nest. The entire *ITS* region of 424 bp length in OTU1 yielded only 12 polymorphic sites which were used for all the population genetics analyses. Here also, we found no sign of population structuring for *Pseudoxylaria* sequences whether the analysis was done with all individuals or using only clone-corrected subpopulations ($p > 0.05$), with very low variation at this subpopulation level (Table S3; Figs. S1C and D). Therefore, as for *Termitomyces* OTU1, the rest of the analysis was performed at the population level, i.e. all 28 sequences were used. Both low G:N ratio and low genotypic diversity (λ) suggested a clonally reproducing *Pseudoxylaria* population. Because of the haploid nature of data, it was not possible to examine HWE for *Pseudoxylaria* OTU1. However, the test for association between loci (rbarD) was significant ($p = 0.001$) when the analysis was done with all individuals (Table 1; Fig. 4C). Because of very low G:N ratio, we repeated the analysis with clone-corrected data which was also significant ($p = 0.001$), rejecting the null hypothesis of no linkage between loci (Table 1; Fig. 4D). PTLPT analysis could not be performed because only one parsimony-informative site was present. These results together are indicative of clonal reproduction in the parasitic fungus. However, this could alternatively be due to recent origin and/or founder effects leading to genetic monomorphism or lack of enough variation in the *ITS* locus of this fungus (Tibayrenc and Ayala, 2012). Therefore, in future, use of other markers like *EF1α* and sampling over a greater geographical area could help in distinguishing between these alternative explanations. Hence, the possibility of sexual reproduction cannot be ruled out in this fungus.

Some human pathogenic fungi, such as *Cryptococcus neoformans, Candida albicans* and *Aspergillus fumigatus*, reproduce clonally with limited evidence of recombination but nonetheless have retained the machinery for sexual reproduction (Heitman, 2006). Heitman (2006) proposed that a clonal population structure may be

a reflection of the adaptation of these fungi to their particular host and environment niches; retaining the ability to reproduce sexually allows them to respond to novel selective pressures such as environmental changes in their host, niches or antimicrobial therapy. *Pseudoxylaria ITS* genotype G7 which was isolated from 22 different nests (Fig. 3B) may thus be the best 'adapted' parasite, reproducing exclusively in a clonal manner. The other less frequent genotypes may represent the less successful genotypes which can undergo sexual reproduction to acquire 'resistant' genes. The only time *Pseudoxylaria* fungi may be able to sexually reproduce inside the nest is when the termite nest is declining. This is when *Pseudoxylaria* species may get the opportunity to grow in an unrestrained fashion, and may reproduce sexually with available compatible mycelia to produce sexual fruit bodies. These fruit bodies are generally visible in abandoned termite nests and may release ascospores into the surrounding environment through the damaged walls of nest mounds neglected by the termites. Then through a mechanism, yet unknown, similar to that which occurs in the mutualistic genus *Termitomyces*, it may find a means to infect new termite nests.

4. Conclusions

Taken together, our results reveal lower OTU and higher genotypic diversity (within the most abundant OTU) for the mutualistic fungus than for the parasitic fungus associated with the fungus-growing termite *O. obesus*. They also suggest a sexual population genetic structure for the mutualistic fungus and indicate a clonal structure for the parasite. Therefore, in this tripartite interaction, the termite host and the mutualist reproduce sexually but the parasite may largely exhibit clonal propagation, even though it may have the potential for sexual reproduction. The parasite may be using clonal reproduction as a strategy to colonize a 'compromised' host, whereas the mutualist enjoys both clonal propagation and sexual reproduction inside and outside the nest respectively.

Acknowledgments

This research was funded by the Council for Industrial and Scientific Research (37(1561)/12/EMR-II), the Ministry of Environment, Forests and Climate Change, the Department of Biotechnology, and the Department of Science and Technology-FIST, Government of India. We are grateful to C Viraktamath and Rashmi Shanbhag for termite identifications, Anagha S J for culturing fungi, Yathiraj Ganesh for field work and to Sunitha Murray for administrative support. We thank S Mahadevan and Praveen Karanth for helpful discussions, and Yuvaraj Ranganathan for introducing us to Inkscape and GIMP. We acknowledge NJ Grünwald and group for the extremely helpful primer available at http://grunwaldlab.github.io/Population_Genetics_in_R/Index.html. We thank Duur Aanen and an anonymous reviewer for greatly improving the manuscript.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.11.003.

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