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The Fungal Diversity in Nests of Three Soil-nesting Ant Species (Hymenoptera: Formicidae) in Pine Forests of Louguantai National Forest Park, China

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Abstract: The abundance and diversity of fungi in nest soil of three ant species, *Camponotus japonicus*, *Formica japonica* and *Tetramorium caespitum* in Louguantai National Forest Park, Qinling Mountains, Shaanxi Prov., China, were studied to determine the fungal biodiversity and biomass in different ant nests and their surrounding ant-unoccupied soil. The results showed that: (1) Fifteen fungal species were isolated from soil samplings in nests of these ants and the surrounding ant-unoccupied soil in total, and the number of fungal species in ant-unoccupied soil was 2.0～3.5 times respectively those in nests of the corresponding ant species. The mean colony-forming units cfu · g^{-1} varied for all soil samplings, in which the mean cfu · g^{-1} in nests of *C. japonicus* was the lowest and showed significantly difference with ant-unoccupied soil ($P<0.05$). (2) All nests had lower fungal diversity index (H') than ant-unoccupied soil, but the fungal evenness index (E) among the ant nests and ant-unoccupied soil had no significant difference ($P>0.05$). (3) All the three ants had special nest fungal communities, but the fungi isolated from their nests had high colony frequency and nearly all could be found in ant-unoccupied soil. The differences of fungal community among the three ants' nests and ant-unoccupied soil might be a result of variations in soil microenvironment and be related to the population, nesting habit and antifungal activities of different ant species.

Key words: ant nest; fungal diversity; soil; *Camponotus japonicus*; *Formica japonica*; *Tetramorium caespitum*; Qinling Mountains

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秦岭楼观台油松林地 3 种土栖性蚂蚁巢内真菌多样性研究

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摘　要:研究比较了秦岭楼观台油松林地日本弓背蚁(*Camponotus japonicus*)、铺道蚁(*Tetramorium caespitum*)及日本黑褐蚁(*Formica japonica*)3 种蚂蚁蚁巢内外土壤真菌多样性的差异。研究结果表明:(1)从 3 种蚂蚁蚁巢及无蚂蚁栖居的土壤样品中共分离到 15 种真菌,无蚂蚁栖居土壤中真菌种类是 3 种蚂蚁巢内真菌种类的 2.0～3.5 倍;日本弓背蚁巢内平均 cfu · g^{-1} 最低,明显低于无蚂蚁栖居的土壤($P<0.05$),另外 2 种蚂蚁巢内及其无蚂蚁栖居的土壤平均 cfu · g^{-1} 差异不明显($P>0.05$)。(2)3 种蚂蚁蚁巢内的真菌多样性指数(H')均显著低于无蚂蚁栖居的土壤($P<0.05$),但在 3 种蚂蚁蚁巢之间无显著差异($P>0.05$);3 种蚂蚁及无蚂蚁栖居的土壤的真菌均匀度指数(E)之间无显著差异($P>0.05$)。(3)3 种蚂蚁巢内真菌种类组成明显不同,形成各自特殊的真菌群落;蚁巢中分布的真菌种类几乎都可发现于无蚂蚁栖居的土壤,但其在蚁巢内的分布均大于

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无蚂蚁栖居的土壤。不同蚂蚁蚁巢内及无蚂蚁栖居的土壤中真菌组成及其多样性的差异与土壤微环境以及不同蚂蚁种群、筑巢习性及其对真菌的防卫机制密切相关。

关键词： 蚁巢；真菌多样性；土壤；日本弓背蚁；日本黑褐蚁；铺道蚁；秦岭

Ants are of ubiquitous, diverse, abundant species in many terrestrial communities worldwide. They have interactions with many different taxa including plants, other insects and invertebrates, and play a prominent role in ecosystem[1-2].

Most ants nests are in soil and may affect the soil biota via numerous pathways. For instance, ant activity and respiration increase moisture and temperature in the surrounding soil[3-4]. Ground-nesting ants increase soil nutrients and change the soil physical and chemical property by carrying aboveground, nutrient-rich materials several centimeters belowground[5-8]. Ants also build belowground galleries and tunnels, thereby disturbing and creating new soil structure[9-10]. Moreover, their nesting activities place them in virtually continuous contact with a vast array of potentially pathogenic microorganisms[11]. As a result of pathogen-related selection pressure, ants employ several tactics as defenses against bacteria and fungi: some ants physically remove fungi and spores from their bodies, other nestmates, and nest chambers[1]; other ants can use chemical defense mechanisms, e. g. using secretions from the metapleural gland to suppress significantly both mycelial growth and spore germination of soil fungi[12-13], thereby controlling fungal infection[14]; leaf cutter ants use antibiotics from actinomycetes to suppress pathogenic fungi[15], and their faecal fluid and mandibular gland secretion can affect microfungal spore germination[16].

The changes in soil conditions related to ants and the mechanical and chemical defenses of ants conceivably influence the microbiota and fungal diversity in ant nests. There were some reports about the soil fungal diversity in China[17-18], but few were on the association between ants and fungi, or on the effects of ant activity on soil fungal diversity.

Herein we have investigated fungi found within nests of three ant species and their surrounding ant-unoccupied soil in pine forests (dominated by *Pinus tabulaeformis*) of the Louguantai National Forest Park in Qinling Mountains, Shaanxi Province, China, which is part of the border of the Palaearctic and Oriental Regions. The objective was to analyze and compare the fungal species richness, diversity, and composition between nest soil and ant-unoccupied soil of these ant species, expecting to reveal the effect of ant activities on fungal distribution, diversity and biomass in soil of the pine forest ecological system.

1 Materials and Methods

Soils from nests of *Camponotus japonicus*, *Formica japonica* and *Tetramorium caespitum* were sampled on 20 July 2008 from the Louguantai National Forest Park in Qinling Mountains, Shaanxi Province, China. All the ants nested in the soil of the pine forests at the altitude of 1 200 m, and their nest entrances were located by following foragers returning from foraging. Voucher specimens of the three ant species were deposited in the Northwest A&F University, China.

We excavated three nests of each ant species. For each soil type (nests of the aforementioned ant species respectively, and ant-unoccupied soil), we wiped out the surface soil and revealed the nest, then used a soil core sampler (∅5 cm) to obtain three samples of the first 8—20 cm of top soil. The soil sampler was rinsed with 75% ethanol between samples to avoid possible cross-contaminations of fungi among nests. The ant-unoccupied soil (about 500 cm apart from sampled ant nests where had no evidence of past occupation by ants) were collected and mixed as one type. Each sample was placed in a plastic bag, sealed, transported in an ice cooler, and returned to the laboratory. All ants and brood that remained in the samples were removed with sterile forceps, and the soil samples were refrigerated overnight at 4 ℃.

For each sample, 10 g of mixed soil were added to 90 mL of deionized water and stirred. From this mixture, 10-folds serial dilutions were prepared. Upon obtaining a final dilution of 10^{-3} for each sample, 0.5 mL of the soil suspension was pipetted onto the surface of a rose Bengal agar plate (∅90 mm). Rose Bengal agar media reduces bacterial growth and limits the growth of invasive fungal species [19]. Agar plates were incubated at room temperature (25 ℃) for 4 d, and an average number of colony-forming units (cfu • g^{-1}) for all soil types were calculated [20]. From each plate, the different colonies were randomly selected and transferred to PDA. These fungal isolates were used to calculate mean colony number, relative colony frequency (percentage of total fungal colonies present) and sample frequency (percentage of samples with fungal species), Shannon-Weaver diversity (H') and evenness (E) indices [21], and coefficients of similarity in each soil type. Isolates that failed to grow or were identified as actinomycetes or yeasts were not included in the analyses. Fungi identification was accomplished after growth according the morphology[22-23]. Data were analyzed by a one-way analysis of variance (ANOVA) followed by a LSD multiple comparison test where warranted with DPS software[24].

2 Results

2.1 Fungal species richness, diversity, evenness, and number of colony forming units in related ant nests and ant-unoccupied soil

We just use the classical culture method with substrate PDA to isolate the fungi related to the nest materials of the three ant species in this study, instead of using molecular procedures in the meantime, since we were aiming mainly to determine preliminary the difference of fungi composition of the nests among the three ant species. Therefore, the fungal species isolated are in all probability fewer than that actually. Fifteen fungal species were isolated from all soil types (Table1, Table 2), among which two species are unknown (didn't produce conidiophores), and others belong to *Rhizopus* (1 species), *Mucor* (3 species), *Aspergillus* (4 species), *Fusarium* (3 species), *Penicillum* (1 species), *Paecilomyces* (1 species), respectively; seven fungal species were isolated from the nests of *C. japonica*, and 4 from *T. caespitum* and *F. japonica*, respectively. However, 14 fungal species were isolated from ant-unoccupied soil. The number of fungal species in ant-unoccupied soil was 2.0～3.5 times those of corresponding nest respectively.

Table 1　Fungal species richness, diversity, evenness, and number of colony forming units in ant nests and ant-unoccupied soil

表 1　蚁巢内与蚁巢外土壤的真菌菌落丰富度、多样性、均匀度和菌落数

	number of fungal species	cfu • g^{-1}	H'	E	soil water content/%
C. japonicus	7	1.93×10^4 b	1.13 (0.025) a	0.87 (0.005) a	18.53
T. caespitum	4	2.93×10^4 ab	1.03 (0.009) a	0.81 (0.008) a	21.83
F. japonica	4	3.50×10^4 a	1.07 (0.031) a	0.86 (0.003) a	19.99
ant-unoccupied soil	14	3.3×10^4 a	2.13 (0.029) b	0.84 (0.009) a	18.82

Note: The different letters in the row indicate significant difference at $P<0.05$ level according to LSD test. Numbers in parentheses are standard errors.

We found that the mean cfu • g^{-1} varied for all soil types (Table1). The nests of *C. japonicus* had a significantly lower number (mean $=1.93\times10^4$, $SE=5.68$) of mean cfu • g^{-1} than the nests of other two ant species and ant-unoccupied soil ($P<0.05$); the mean cfu • g^{-1} in nests of *F. japonica* (mean $=3.5\times10^4$, $SE=5.0$) was nearly equal to that of ant-unoccupied soil (mean $=3.43\times10^4$, $SE=6.5$) and was slightly higher than nests of *T. caespitum* (mean $=2.93\times10^4$, $SE=6.11$), but the differences were not significant ($P>0.05$).

The fungal diversity indices (H') in nests of the three ant species were all significantly lower than that of ant-unoccupied soil ($F_{3,8}=47.49$, $d_f=3$, $P=0.00$), but which among the nests of the three ant species were not significantly different ($F_{2,6}=0.878$, $d_f=2$, $P=0.478$) (Table1). Similarly, the species evenness indices (E) of all soil types were not significantly different either ($F_{3,8}=0.259$, $d_f=3$, $P=0.85$) (Table 1).

Table **2** Colony number, colony frequency (%) and sample frequency (%) of fungal species found in ant nests and ant-unoccupied soil

表 2 蚁巢和蚁巢外土壤的真菌种类数、菌落丰富度和抽样频率

No.	Fungal species	Nest soil of *C. japonicus*		Nest soil of *T. caespitum*		Nest soil of *F. japonica*		Ant-unoccupied		*sf*
		cn	*cf*	*cn*	*cf*	*cn*	*cf*	*cn*	*cf*	
1	*Rhizopus nigricans*	3	5.17			43	40.95	5.5	2.63	11.39
2	*Mucor globosus*	17	29.31	51	64.15			33	13.37	22.35
3	*Aspergillus fumigatus* var. *fumigatus*	15	25.86					28	13.37	9.51
4	Unknown species 1							14	6.68	3.10
5	*Fusarium sambucinum*	5	8.62					1	0.48	1.33
6	*Penicillum restrictum*	7	12.07							1.55
7	*M. rouxii*	3	5.17					2	0.95	1.11
8	*A. alliaceus*	8	13.79			29	27.62	68	32.46	23.23
9	*F. neoceras*			12	15.09			5	2.39	3.76
10	*F. coeruleum*			8.5	10.69	28	26.67	12	5.73	10.73
11	*A. penicillioides*			8	10.06			3	1.43	2.43
12	*Mucor jansseni*							6	2.86	1.33
13	*Paecilomyces* sp.					5	4.76	16	7.64	4.65
14	*A. clavatonanicus*							8	3.82	1.77
15	Unknown species 2							8	3.82	1.77
	Number of species	7		4		4		14		
	Total number of colonies	58		79.5		105		209.5		452

Notes: *cn* = number of colony, *cf* = colony frequency (%), *sf* = sample frequency (%).

2.2 Fungal community similarity of ant nests and ant-unoccupied soil

The number and composition of the fungi in the three ant nests were different. There was few overlap in fungal species among them, and each ant species had special fungal community. In addition, the fungi isolated from their nests had high colony frequency and nearly all could be found in ant-unoccupied soil except *Penicillum restrictum* which was only found in *C. japonicus* nests (Table 2). Based on calculated coefficients of fungal community similarity (Table 3), the similarities among the four soil types were very low, in which *C. japonicus* shared only 0.4% of fungal species with ant-unoccupied soil and 0.1% with *T. caespitum*, and the other two ant species both shared only 0.29 % fungal species with ant-unoccupied soil.

Table **3** The fungal community similarity of ant nests and ant-unoccupied soil

表 3 蚁巢和巢外土壤的真菌群落相似性

	Nest soil of *C. japonicus*	Nest soil of *T. caespitum*	Nest soil of *F. japonica*	Nest soil of Ant-unoccupied soil
C. japonicus	1			
T. caespitum	0.1	1		
F. japonica	0.25	0.14	1	
ant-unoccupied soil	0.4	0.29	0.29	1

3 Discussion

The mean cfu · g^{-1} varied for the nests of three ant species, but the difference was not prominent ($P>0.05$), except mean cfu · g^{-1} in *C. japonicus* which was the lowest and significantly different to the others. This result is not consistent with other related studies, e.g. Czerwiñ ski *et al*. found a 10-fold increase in the number of cfu · g^{-1} in *Lasius niger* and a three-fold increase in *Myrmica* sp. mounds in comparison with uninhabited soil[25]; Zettler[26] *et al*. found that fire ant (*Solenopsis invicta*) mound contained 19 times more fungal colonies than adjacent soil. The three ant species in our study all nest underground, but in comparison with *Lasius niger* Myrmica sp. and fire ant, they don′t make discernable mounds, so the environment conditions within and outside their nests might not vary as much as *L. niger Myrmica* sp. and fire ant. Therefore, these differences of fungal community among the nests of different ant species are closely relate to the nesting behaviors and nest environment of different ant species.

In our study, we found that the fungal species richness and diversity indes (H') in the involved ant nests were distinctly lower than these in ant-unoccupied soil ($P<0.05$), but the diversity index (H') and evenness index (E) had no distinct difference among the involved ant nests ($P>0.05$). Although all fungal species isolated from involved ant nests could be found in ant-unoccupied soil, but with lower colony frequencies. This result is consistent with those of Zettler[26] *et al*. who found that both fire ant mounds and nests of *Aphaenogaster texana carolinensis* Wheeler (native ant of Clemson, SC, USA) had lower fungal species richness and diversity than non-mound soil (fire ant mounds was reduced by over 50% in comparison with the surrounding soil). Ba[27] *et al*. found that more yeast species were isolated from non-mound soil than brood chamber soil of fire ant, and the mean cfu $\cdot$ g^{-1} of soil was greater than in brood chamber soil. Therefore, those researches showed that fire ants alter the community composition and the relative abundance of fungi within mounds due to creation of unique microhabitat patches, and these high densities of limited numbers of fungal species in fire ant mounds possibly indicate that only some species are tolerant to and thrive in nest conditions, or alternatively, ants might not selectively remove these fungi from their nests.

The difference of fungal community among the nests of the three ant species and ant-unoccupied soil investigated here also might be a result of variations of soil microenvironment, e. g. variations of soil pH, temperature, humidity, nutrients and aeration in these microhabitats. Environmental heterogeneity exerts a powerful influence on the distribution of organisms, their interactions and their adaptations[28]. Alternatively, these fungal differences might be due to the presence of antimicrobial defenses in ants and degrees of fungal resistance to these defenses. For example, secretions from metapleural glands of *Aspergillus niger* can significantly reduce hyphal growth in some fungi, but one fungal species, *Metarhizium brunneum*, showed resistance to metapleural secretions[12]. Alkaloids from venom glands of *S. invicta* inhibit conidial germination, but they have little effect on hyphae[29]. Regarding to the ant species studied here, *F. japonica* and *T. caespitum* do have metapleural glands, while *C. japonicus* doesn't have metapleural glands; and more experiments are needed to verify if they have the same antimicrobial defense as some other ants or have special ways to affect fungal diversity of their nests.

Exactly, the composition of fungal species in different ant nests also may relate closely with the species, individual size, population, nesting habit and their antifungal activities. In our study, both *C. japonicus* and *F. japonica* belong to Formicinae, and the worker of *C. japonicus* are the biggest in size (about 7.4－13.8 mm in length) and its population could be 4 000 in number; for *F. japonica*, its worker size is about 5.4-7.6 mm in length and the population could be 3 000 in number. *Tetramorium caespitum* belongs to Mymicinae; its worker size is very small (about 2.6－3.9 mm in length) but has bigger population[9]. These differences may lead to different effects on their nest soil. In addition, *C. japonicus* and *F. japonica*, with nest depth of 0.88 m on average, always put dust outside, so their nests are relatively clean. In comparison, nests of *T. caespitum* are shallow and usually mixed with some leaf litter. Therefore, the dissimilarity of fungal species between the nests of *T. caespitum* and the other two ant species may also related to the differences of the nest constructions.

The soil fungal diversity can be affected by many factors, such as season, vegetable, climate, the physical and chemical character of soil, etc.[18]. However, our study was limited to only one locality (the Louguantai National Forest Park in Qinling Mountains, Shaanxi Province) at one selected date. Therefore, more studies are needed to value the limitative effects of ants on the fungal diversity in ant-occupied soil, and to help further understanding of the ant-fungus associations in the related pine forest ecosystem we identified.

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