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# Variation and diversity of symbiotic protist composition in the damp-wood termite *Hodotermopsis sjoestedti*

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## SUMMARY

It is well recognized that composition of termite symbiotic protist community generally show host-species specificity at species-to-species level. On the other hand, studies focused on population or colony level variations of the protist composition are few, although such studies are necessary for understanding the termite-protist mutualism. In this study we investigated symbiotic protist fauna of 34 *Hodotermopsis sjoestedti* nests collected from five islands in the Ryukyu Archipelago, Taiwan Is., and southern part of Chinese continent. Thirteen parabasalid species and six oxymonad species, which were reported from the host termite collected in Yakushima Is., were recognized in this study. Four parabasalids were identified as recently described species from *H. sjoestedti* collected in Vietnam. The fauna was the most species-rich among the termite hosts studied to date, possibly reflecting large host body size. The prevalence rates of the symbiotic protist species are generally high, 100% in all populations for all protist species, except *Gen. A* sp. and *Hoplonympha* sp. All host nests had identical protist species composition except for the presence or absence of *Gen. A* sp. Two host populations lacked this species. In Yakushima Is., both types of termite nests were found, which exhibited mutually exclusive distribution. The distribution pattern suggests that the host termite is a multiple-site nester and nest-budding occurs in the field.

Key words: Symbiosis, Parabasalia, Oxymonadida, Protist community

## INTRODUCTION

Symbiotic protist community in hindguts of termites is one of the textbook examples of wood-

degrading symbiosis (Cleveland, 1923; Inoue et al., 2000; Brune and Ohkuma, 2011; Ohkuma and Brune, 2011). The symbionts are consisted of two lineages of anaerobic protists Parabasalia and Oxymonadida that lack mitochondria and can not survive outside the host digestive tracts (Brugerolle and Radek, 2006). The host termites are social insects and live in large family group called

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“colony”, making up spatially structured aggregation of community of the symbiotic protists. A new host colony is founded by a pair of male and female alates (*i.e.*, winged reproductive individuals, called king and queen), and they succeed the protist symbionts of mother nests.

From the viewpoint of community ecology, the termite symbiont community is unique among symbiont (or parasite) communities in animal digestive tracts because the species composition generally shows strong host-species specificity: a certain set of protist species are found from digestive tracts of a termite species (Kirby, 1934; Honigberg, 1970; Inoue et al., 2000; Kitade, 2004). The mode of symbiont transmission through generations should lead to co-speciation between the hosts and the symbionts and thus the specificity of the fauna. The co-speciation was verified for a parabasalid genus *Pseudotrichonympha* spp. and host termites of the family Rhinotermitidae using molecular phylogenetic analysis (Noda et al., 2007; Lo and Eggleton, 2011).

Although this species-to-species level host specificity is well recognized and described in taxonomic papers (Yamin, 1979), taxonomists also noticed that sometimes a termite individuals or, rarely a whole colony, lack a particular protist species (Honigberg, 1970). However, most of the reports were fragmentary and ecological studies were few that gave a fine description of colony- or population-level variations of the protist species composition. A detailed study was carried out for the symbiont composition of *Reticulitermes* (Rhinotermitidae), a common termite genus in Japan, based on the survey of the 84 field colonies of six host species in the Japan Archipelago (Kitade and Matsumoto, 1993). The study revealed that at species-to-species level, the host termites certainly exhibited strict host specificity. However, the faunae in host individuals were not absolutely uniform and the mean prevalence of each protist species in a host species ranged from 0.68 to 1.0. Each host species possessed species-specific

“basic symbiont species composition”, and from this composition, a small fraction of individuals or colonies lacked one or two symbiont species. For other termite taxa, there is no reliable description about the variation of the symbiont fauna, while such ecological basis of the termite-symbiotic protist community is important for understanding the establishment and maintenance processes of the symbiotic protist community and evolution of mutualism.

In this study we investigated symbiotic protist fauna of *Hodotermopsis sjoestedti* Holmgren, a single member of the genus *Hodotermopsis* in the family Termopsidae, which is distributed in East Asia including southern part of Chinese continent, Taiwan Is., and some islands in the northern Ryukyu Archipelago (Matsumoto and Hirono, 1985). This species is one of the host insects of parabasalid and oxymonad protists with the largest body size. Like other members of the Termopsidae, *Hodotermopsis* has been regarded as a “single-site nester” whose colony nests in a single large dead log or stump (Matsumoto and Hirono, 1985). However, recent study of termite phylogeny based on sequences of multiple genes did not support the monophyly of Termopsidae. The inferred phylogeny suggested that *Hodotermopsis* formed a clade with the members of Hodotermitidae, although support of the topology is not strong (Inward et al., 2007a).

From *H. sjoestedti* in Japan, 19 species of the symbiotic parabasalids and oxymonads were tentatively reported by Kitade et al. (1993). Brugerolle and Bordereau (2004) and Brugerolle (2005) observed ultrastructure of the parabasalid symbionts and described five species from materials in Vietnam. Based on these preceding studies, we focused on investigation of the variations in symbiont species composition between termite colonies and between regional populations using nests from Ryukyu Archipelago, Taiwan Is., and Guangdong prov., Continental China. To examine as many fresh termite nests and individuals as possible and

to grasp characteristics of the field community, we did not investigate population size of each symbiont species (*i.e.*, community structure). The community structure is sensitive to changes in environmental conditions and requires much longer time for the investigation.

## MATERIALS AND METHODS

### *Sample collection*

We collected host termite nests during 1990–2010 from all the islands in the Ryukyu Archipelago, Japan, where *H. sjoestedti* was reported: 15 nests from Yakushima Is., two from Tanegashima Is., five from Nakanoshima Is., four from Amamioshima Is., and five from Tokunoshima Is. These nests were collected from dead logs in laurel forests, and in Yakushima Is., we mapped 14 nests collected in Onoaida in Nov. 1991. The termites, together with nest wood, were brought back to the laboratory and maintained individually in plastic boxes at 22 or 25°C under all dark condition until investigation of the intestinal protists. We also observed termite symbionts from two nests from Nanshanxi, Nantou county, Taiwan Is., Republic of China and Shixhin, Guandong province, Continental China, just after they were collected in the field. The host termite distributed in the Ryukyu Archipelago and Shixing was formerly classified as *H. japonica* and *H. yui*.

### *Examination of protist composition*

Preceding the investigation of the protist composition, we made and observed protargol-stained specimens of the protists using one representative termite from each locality in Japan following Kitade et al. (1997). Based on the specimens, we listed the protist species and carefully compared the morphology of them with living materials in order to identify all the symbiont species in living state.

To investigate the variation of protist composition, for each termite nest, we examined presence

or absence of the protist species for ten termites individually. The examined termites were fifth- or later-instar larvae and thought to function as main workforce in the colony. We pulled out the intestine of the living insects with forceps, dissected it in 0.6% NaCl solution, and suspended the intestinal contents. Then we dispensed intestinal fluid onto several glass slides and observed all of them by differential interference microscope (BX-50, Olympus).

For the samples of Taiwan Is. and Shixhin, protist composition was examined by observation of all intestinal contents using a portable phase-contrast microscope (DSM-II, Daiko Science). For these samples, protargol specimens were examined after the living samples were investigated.

### *Statistic analysis*

Based on the protist composition data of ten termites from each of termite nests, we calculated mean prevalence value of each of seven populations. Standard deviations (SD) of the prevalence values between colonies were also calculated for each population.

We carried out Mantel test (Mantel, 1967) to examine whether the host nests of the different symbiotic protist composition types exhibited mutually exclusive distribution in Onoaida, Yakushima Is. A matrix of geographical distance and that of ‘distance of protist composition type’ were calculated between all termite nests mapped in Onoaida. As the distance of protist type, 0 and 1 were respectively assigned for nest pairs of same and different composition types (A and B, see results and Table 1). Then a Mantel statistic  $Z_M$  was calculated as the sum of cross products of the corresponding elements,  $x$  and  $y$ , of the two matrices:

$$Z_M = \sum_{i=1}^{n-1} \sum_{j=i+1}^n x_{ij} y_{ij}$$

where  $i$  and  $j$  are row and column indices and  $n$  is number of the nests, respectively. Significance of the correlation was tested by comparing the origi-

nal  $Z_M$  value with the frequency distribution of  $Z_M$  values calculated from 999 pairs of the matrices, which were produced by random element interchanges of the original matrices. The test was carried out with R ver. 2.9.2 (The R project for statistical computing, <http://www.r-project.org/>) and “vegan” program package.

## RESULTS

All of the 13 parabasalid species and the six oxymonad species, reported from *H. sjoestedti* from Yakushima Is. in the Ryukyu Archipelago (Kitade et al., 1997), were recognized from the materials of this study (Table 1).

Of these species, four parabasalids were recently described species from *H. sjoestedti* in Vi-

etnam by Brugerolle and Bordereau (2004: *Holomastigotes lanceolata*) and Brugerolle (2005: *Microjoenia minima*, *Spirotrichonympha cincta*, *Spiromyxa oblonga*, and *S. obtusa*). Additionally, one *Eucomonympha* and three *Trichonympha* species reported by Brugerolle and Bordereau (2004) corresponded well to the generic and specific composition of symbionts recognized in this study.

The protist fauna found in this study includes three parabasalid species that had similar morphology to *Trichomonoides trypanoides* (Duboscq and Grassé), *Holomastigotes elongatum* Grassi and *Hoplonympha natator* Light, recently reported from *H. sjoestedti* in Vietnam (Brugerolle, 2005). However, we tentatively did not assign these specific names to our materials: the latter three spe-

Table 1. Prevalence of each protist species

Symbiont Species	Population / Number of termites (nests) examined							Total 340 (34)
	YK 150 (15)	TN 20 (2)	NK 50 (5)	AM 40 (4)	TK 50 (5)	NSX 20 (2)	SHX 10 (1)	
<b>Parabasalida</b>								
<i>Trichomonoides</i> sp. <sup>1)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Gen. A</i> sp.	0.55 ± 0.49	1.00	-	1.00	1.00	1.00	-	0.66 ± 0.47
<i>Microjoenia minima</i>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Spirotrichonympha cincta</i>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Spiromyxa oblonga</i> <sup>2)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Spiromyxa obtusa</i> <sup>3)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Holomastigotes lanceolata</i> <sup>4)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Holomastigotes</i> sp. <sup>5)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Hoplonympha</i> sp.	0.95 ± 0.18	1.00	0.82 ± 0.40	1.00	0.92 ± 0.18	1.00	1.00	0.94 ± 0.20
<i>Eucomonympha</i> sp.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Trichonympha</i> sp. 1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Trichonympha</i> sp. 2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Trichonympha</i> sp. 3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<b>Oxymonadida</b>								
<i>Pyrsonympha</i> sp. 1 <sup>6)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Pyrsonympha</i> sp. 2 <sup>6)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Dinenympha</i> sp. 1 <sup>7)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Dinenympha</i> sp. 2 <sup>7)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Dinenympha</i> sp. 3 <sup>7)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>Gen. B</i> sp. <sup>8)</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Basic protist composition type	A(8)*, B(7)*	A	B	A	A	A	B	

Each value is represented by mean ± SD. YK: Yakushima Is.; TN: Tanegashima Is.; NK: Nakanoshima Is.; AM: Amami-oshima Is.; TK: Tokunoshima Is.; NSX: Nanshanxi; SHX: Shixing. \*: No. of colonies with each protist composition type. 1)-8) corresponds to the species listed in Kitade et al. (1997), <sup>1)</sup>: *Trichomonas* sp.; <sup>2)</sup>: *S.* sp. 1; <sup>3)</sup>: *S.* sp. 2; <sup>4)</sup>: *H.* sp. 1; <sup>5)</sup>: *H.* sp. 2; <sup>6)</sup>: *Pyrsonympha* (*Pyrsonympha*) sp. 1-2; <sup>7)</sup>: *P.* (*Dinenympha*) sp. 1-3; <sup>8)</sup>: *P.* (*Subgen. A*) sp.

cies were originally described as symbionts of distantly related termites to *Hodotermopsis*, (*Reticulitermes* spp. and *Paraneotermes simplicicornis*), and *Hoplonympha* sp. found from *H. sjoestedti* populations in this study often exhibited much larger body length than *H. natator*. Brugerolle and Bordereau (2004) reported *Trichomitopsis termopsidis* (Cleveland), a symbiont of termite genus *Zootermopsis* spp. (Termopsidae), from *H. sjoestedti* in Vietnam. In this study, however, *T. termopsidis* was not found but we recognized *Gen. A* sp. (see Kitade et al., 1997). Examination of protargol specimens revealed that this species had typical morphological character states of genus *Trichomitopsis* and similar morphology to *T. termopsidis*. On the other hand, *Gen. A* sp. is, besides a single nucleus and axostyle, equipped with two karyomastigonts (Kitade et al., 1997) which *Trichomitopsis* and other typical Trichomonadid species (*sensu* Brugerolle and Patterson, 2001) have one.

Six oxymonad species reported by Kitade et al. (1997) were definitely recognized by morphology. For *Dinenympha* and *Pyrsonympha*, however, the fauna might include 1-2 unrecognized species. *Gen. B* sp. could not be assigned to the any extant genus (see Kitade et al., 1997).

At the level of termite nests (colonies), two types of basic protist compositions were recognized: the composition composed of the 19 protist species (hereafter, composition type A) and that lacked only one species, *Gen. A* sp., from the type A composition (composition type B). All investigated nests in Amamioshima Is., Tokunoshima Is. and Nanshanxi had type A and those in Nakanoshima Is. ( $N = 5$ ) and Shixhin ( $N = 1$ ) had type B. In Yakushima Is., both nests with composition type A ( $N = 8$ ) and those with type B ( $N = 7$ ) were found. The prevalence rates of the symbiotic protist species were generally high, 100% in all populations for all protist species, except *Gen. A* sp. and *Hoplonympha* sp. The prevalence of *Gen. A* sp. was 100% in Tokunoshima Is., Amamioshima

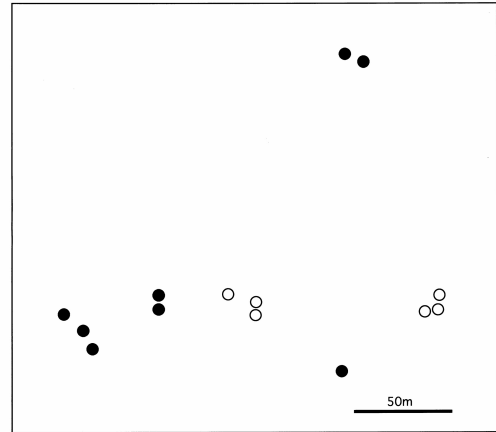


Fig. 1. Distribution of *H. sjoestedti* nests found in 1991 in the study site in Onoaida, Yakushima Is. Closed and open circles represent nests with basic symbiont composition type A and B, respectively (see Table 1).

Is. and Nanshanxi, while it was much lower in Nakanoshima Is. (0%), Shixhin (0%), and Yakushima Is. (55.3%). One nest was found in each of three host populations containing termites without *Hoplonympha* sp.: Yakushima Is. (prevalence: 95.3%), Nakanoshima Is. (82.0%), and Tokunoshima Is. (92.0%).

In the sampling site in Onoaida, Yakushima Is., both type of host nests with and without *Gen. A* sp. were found (Fig. 1). The both type of nests exhibited significantly mutually exclusive distribution ( $N = 14$ , Mantel test,  $P = 0.041$ ).

## DISCUSSION

The high prevalence rate of each symbiotic protist species in *H. sjoestedti* is the common feature with *Reticulitermes* host termites investigated by Kitade and Matsumoto (1993), and probably the general characteristic of termite protist community. As with *Reticulitermes* hosts, *H. sjoestedti* has basic symbiont composition composed of 19 species, and from this composition, a particular local population or individual lack one or two symbiont species. Such high host specificity found

in the termite protists is rare among the intestinal parasite/symbiont communities of metazoans (e.g. Esch et al., 1990). Together with the transmission mode of protists, the specificity probably reflect close mutualistic relationships between the hosts and the multiple symbionts including intestinal prokaryotes, through complex metabolic system involved in wood degradation (Brune and Ohkuma, 2011).

The symbiotic protist fauna of *H. sjoestedti* is the most species-rich among the termites studied to date (Kitade et al., 1997; Yamin, 1979), except for wood feeding cockroach genus *Cryptocercus*, which is now recognized as sister taxon of termites based on the molecular phylogenetic analyses (Lo et al., 2000; Inward et al., 2007b). The prevalence rates of the protist species constituting the basic symbiont composition of *H. sjoestedti*, 100% except for two parabasalid species, are rather higher than those of *Reticulitermes* spp. (Kitade and Matsumoto, 1993) sympatrically distributed in the sampling localities of this study and often coexisted in the same log (Kitade, 2004). Degrees of diversity and stability of the protist communities are likely to be explained by host body size, as expected from the theory of island biogeography (MacArthur and Wilson, 1967). The much larger body size and hindgut volume of *Hodotermopsis* (*H. sjoestedti* soldier mean head width: 4.69 mm,  $N = 143$ , Huang et al., 2000) probably allow the larger population size of each symbiont species and thus diverse and stable symbiont community structure than *Reticulitermes* (*R. speratus speratus* soldier mean head width: 1.18 mm,  $N = 25$ , Takematsu, 1999). Similarly, large body size of *Cryptocercus* (*C. punctulatus* adult mean head width: 4.76 mm,  $N = 44$ , Nalepa et al., 2008) probably enables the maintenance of the species-rich symbiont community (*C. punctulatus*: 23 species were recognized by Cleveland et al., 1934).

The Nakanoshima and Shixhin populations of *H. sjoestedti* lacked the symbiont species *Gen A* sp. This population-level difference of the species

composition does not show correspondence to the phylogeny among the populations inferred from mitochondrial gene sequences (Maekawa et al., 1998). The most parsimonious explanation based on the host phylogeny is that the common ancestor of *H. sjoestedti* harbored *Gen A* sp. and in some populations lack of the symbiont species occurred independently. This symbiont species probably has characteristics that tend to trigger colony- or population-level local extinction.

In Yakushima Is., both types of *H. sjoestedti* nests coexist that harbor and lack *Gen A* sp., showing mutually exclusive distribution. These facts lead following suggestions concerning ecology and reproductive mode of the host termite. First, *H. sjoestedti* is not a single-site nester, but a “multiple-site nester” whose colony simultaneously utilizes multiple logs as nests and feeding sites. It is presumable that neighboring host nests with same protist composition type are owned by a single colony. Some reports of field studies that several nests of this termite species lacked reproductive castes (king and queen) (Matsumoto and Hirono, 1985; Miura et al., 2000) possibly support our suggestion. Moreover, although all termites in the family Termopsidae, including *H. sjoestedti*, has been recognized as a single-site nester, a recent molecular phylogenetic study suggests that *Hodotermopsis* does not form monophyletic group with other Termopsid genera, but is closely related to genera in the Hodotermitidae (Inward et al., 2007a).

Second, outbreeding by alate reproductive pair (king and queen) may not be a dominant reproduction mode of *H. sjoestedti*. When an alate pair establishes a new nest by outbreeding, the colony members succeed the symbiotic protist fauna of the mother colonies of the alates (Wakui, unpublished data). In a normal termite population where the outbreeding is dominant, even if a symbiont species disappeared in a termite colony, the incomplete symbiont fauna of the alates from the colony must be complemented by their mates and



the alate pairs can produce new colonies with complete fauna. The high intra-populational variation and patterns of distribution of the two types of fauna in Yakushima Is. suggest that *H. sjoestedti* colonies mainly multiply through unusual mode of reproduction, possibly by colony budding and inbreeding by neotenic reproductives. These possibilities should be verified by ecological genetic studies of the host termite colonies using high-resolution codominant DNA markers.

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