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Effects of geographical and dietary variation on the symbiotic flagellate protists communities of the subterranean termite *Reticulitermes grassei* Clément

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Effects of geographical and dietary variation on the symbiotic flagellate protists communities of the subterranean termite *Reticulitermes grassei* Clément

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ABSTRACT

Despite their importance on diverse ecosystems, termites may also be considered severe pests of wood in service, and also as agricultural and forestry pests.
Subterranean termites' ability to digest lignocellulose relies not only on their digestive tract physiology, but also on the symbiotic relationships established with flagellate protists and bacteria. In this tripartite lignocellulolytic system, the termite contribute with endogenous cellulases and mechanical processing, flagellate protists phagocyte the wood particles and digest them, and prokaryotes have, among others, an important role in maintaining the physical-chemical equilibrium inside the termite hindgut. The flagellate protist community living inside the termites is rather diverse, as there is a strong division of labour among them to accomplish the intricate process of lignocellulose digestion.
The objectives of this work were to: 1) investigate the changes in flagellate protists communities of the termite *Reticulitermes grassei* in different locations; 2) test the possible effect of different laboratorial diets on diversity and abundance of the flagellate protists.
R. grassei termites were captured in four different locations (Évora, Faial Island, Leiria and Sesimbra), in Portugal, and their symbiotic flagellate protist community diversity and abundance was evaluated. Termites belonging to the same colony were submitted to six different diets (natural diet, pine wood, European beech, thermally modified beech, cellulose and starvation) and after the trials their flagellate protist community was also evaluated.
The differences between termite colonies from different locations may not be denied, although not considered to be significant. Similar flagellate protists communities were found on non-treated sound woods, while cellulose fed and starving termites had significantly different communities. The flagellate protists community of untreated beech and thermally modified beech fed termites were considered to be significantly different, with three morphotypes missing in the treated wood fed termites.
Although the effects of geographical location were not considered significant, the laboratory diets caused major adaptations of the flagellate protists communities. The termite symbiotic flagellate protists community is a dynamic assemblage able to adapt to different conditions and diets.

Keywords: subterranean termites, symbiotic flagellate protists, lignocellulose digestion

1. INTRODUCTION

Despite their importance on diverse ecosystems, termites may also be considered severe pests of wood in service, and also as agricultural and forestry pests (Rouland-Lefèvre 2011). Subterranean termites represent approximately 80% of the economically important species in this context (Nobre and Nunes 2007, Rust and Su 2012). Factors as the continuous growth of human population and climate changing may favour the establishment or growing of populations of termites. Therefore, the need of finding efficient prevention and control methods for subterranean termite pests is crucial. Management strategies that take account of the biology and behavior of the species have the potential to be more efficient and sustainable. Understanding how termites feed and obtain their nutrients from food, can unveil valuable tools to be used in innovative control strategies.

Termites ability to efficiently digest cellulose rely not only on chemical features (cellulolytic enzymes), but also on the digestive tract physiological properties. Termite digestive tract is composed of different parts: mouth, salivary glands, foregut, midgut and hindgut, and each part have a specific function on lignocellulose breakdown. Termites established symbiosis with other organisms: flagellate protists and bacteria, harboured in the dilated portion of the hindgut, the paunch, were the bulk of chemical reactions of lignocellulose breakdown occur. In this tripartite lignocellulolytic system, in a simplistic approach, the termite contribute with endogenous cellulases and mechanical processing, flagellate protists phagocyte the wood particles and digest them, and prokaryotes have, among others, an important role in maintaining the physical-chemical equilibrium inside the termite hindgut.

Within a termite colony and amongst castes, a rather homogeneous gut symbiotic fauna is expected, as termites rely on horizontal transmission of hindgut symbionts to recover the hindgut symbiotic community after moulting. The recovery of the symbiotic fauna is done by proctodeal or stomodeal trophallaxis and grooming behavior. The obligatory vertical mode of transmission of the gut symbiotic fauna to the next generation likely determines the gut symbiotic structure associated to termite species colonies leading thus to higher levels of host-symbiont specificity (Hongoh *et al*. 2005, Noda *et al*. 2007, Husseneder 2010). Recently the term holobiont has been accepted to refer to the host and its microbiota as a whole unit, able to live, develop, survive and evolve together (Rosenberg and Zilber-Rosenberg 2013).

Flagellated protists are part of the unicellular eukaryotes belonging to two separate lineages: the order Oxymonadida (Phylum Preaxostyla) and the Phylum Parabasalia (Brugerolle 1991, Cepicka *et al*. 2010, Adl *et al*. 2012). The diversity of flagellate protists fauna associated to lower termites might be explained by a strong division of labour to accomplish the intricate process of lignocellulose digestion: a species or group of species acts on specific phases of this process (Yoshimura *et al*. 1996, Inoue *et al.* 1997, Todaka *et al*. 2007, Raychoudhury *et al*. 2013).

Within this are the particular objectives of this work were to: 1) investigate the changes in flagellate protists communities of the termite *Reticulitermes grassei* Clément (Blattodea: Isoptera: Rhinotermitidae) in different locations, in Portugal; 2) test the possible effect of different laboratorial diets on diversity and abundance of the flagellate protists.

2. EXPERIMENTAL METHODS

2.1. Geographic variation

Reticulitermes grassei termites were captured in Portugal, in four different locations: 1) in a house backyard in Évora city; 2) in a house backyard in Horta city, located in Faial Island (Azores); 3) in a forest of *Pinus pinaster* Aiton in Leiria district; and 4) in a forest of *P. pinaster* in Sesimbra district.

Termites were collected from decayed logs of pine in the forest, or from a wooden box and old pallets on the backyards, and brought to laboratory for further analysis. Twenty termites from each location were evaluated in terms of protists diversity and abundance by direct observation under a microscope, and for flagellate protists quantification a haemacytometer was used. Only termite workers in the 4th instar were used. Termites were rinsed briefly in Trager Medium U buffer (Trager 1934) to remove surface contaminants. An incision was made in the last abdominal segment of the worker and the digestive tract was pulled out using a fine-tipped forceps. The anterior part of the digestive tract was cut out and the posterior part was suspended in 25 μL of Trager Medium U buffer. The hindgut was cut open and a slight homogenization to release its contents was done. The termite tissue was removed and the remaining solution was loaded in a haemacytometer (8μL) and observed in a light microscope (x40). Flagellate protists were identified to different morphotypes and counted. For each termite, five small squares, of 0.00625 mm^3 of volume, were chosen randomly for counting the protists. The calculations for the number of flagellate protists were estimated to the observed area of the haemacytometer according with the formula:

$$\frac{\text{No. FP} * V_{OAH}}{V_{SSq}}$$

No. FP is the number of flagellate protists observed; V_{OAH} is the volume of the haemacytometer; and V_{SSq} is the volume of the small squares where the flagellate protists were counted.

2.2. Dietary variation

In parallel, termites from location 4 (Sesimbra) were submitted to six different diets: natural diet (pieces of the original wood from where they were collected), pine wood (*Pinus pinaster*), European beech (*Fagus sylvatica* L.), thermally modified beech – beech TMT (submitted to 180ºC during 4 hours), cellulose (cellulose powder mixed with deionized water) and starving (no source of cellulose offered to the termites). The trials were established according with EN117 (2005), with some adaptations. Three replicates per each diet category were set, with 250 workers each and 1-5% of soldiers and pre-alates in order to maintain the colony natural ratio of each caste. The test replicates were maintained in a conditioned room at 24 ± 1ºC temperature and 80 ± 5% relative humidity.

The time of exposure was of 14 days, except for starving termites, which were left to starving during six days. In the end of the trials, survival rate of termite workers and cellulose source mass loss (if applied) were evaluated. The same methods of termite dissection and flagellate protists identification and quantification described above (2.1.) were applied after the trials.

3. RESULTS AND DISCUSSION

3.1. Geographic variation

The flagellate protists were preliminarily identified based on morphological characters and separated to 14 morphotypes (Table 1). Ideally further investigation with molecular markers should be pursued in order to establish robust identification based on both morphological and molecular data (Harper *et al*. 2009, Tai *et al*. 2013). The results of flagellate protists counting are stated in Table 2.

A paired T-test was performed between the termite colonies from different locations, to investigate if the null hypothesis (flagellate protists diversity and abundance is not significantly different among different termite colonies locations; $p<0.05$) was true and the results obtained were not significant for any of the paired tests, therefore the null hypothesis was accepted.

The major part of gut symbionts are considered to be autochthonous and probably co-evolving with termite species (Hongoh *et al.* 2005, Noda *et al.* 2007). The colonization of new habitats or

the proximity of other termite species do not seem to have a significant influence on the symbiotic fauna of lower termites, not only because of the necessity of maintaining all the actors playing the different roles of the strong labour division within the symbiotic flagellate protists during the lignocellulosic degradation process, but also due to the defensive mechanisms of gut symbionts towards external agents (Chouvenc and Su 2012). In addition, it is proved that within a termite colony and amongst castes, generally a homogeneous gut symbiotic fauna should be present, as termites recover the symbiotic fauna discarded after the moult through horizontal transmission.

Table 1: Flagellate protists identification to morphotypes based on morphological characters.

	Phylum	Class	Order	Family	Genus	Species
n1	Parabasalia	Trychonymphea	Trichonymphida	Trichonymphidae	*Trichonympha*	*T. agilis*
n2	Preaxostyla	-	Oxymonadida	-	*Pyrsonympha*	
n3	Preaxostyla	-	Oxymonadida	-	*Dinenympha*	*D. gracilis*
n4	Parabasalia	Spirotrichonymphea	Spirotrichonymphida	Holomastigotoididae	*Holomastigotes*	*H. elongatum*
n5	Preaxostyla	-	Oxymonadida	-	*Dinenympha*	*D. fimbriata*
n6	Parabasalia	Hypotrichomonadea	Hypotrichomonadida	Hypotrichomonadidae	*Trichomitus or Trichomitopsis*	
n7	Preaxostyla	-	Oxymonadida	-	*Pyrsonympha*	
n8	Parabasalia	Spirotrichonymphea	Spirotrichonymphida	Holomastigotoididae	*Microjoenia*	
n9	Parabasalia	Trychonymphea	Trichonymphida	Teranymphidae	*Pseudotrichonympha*	
n12	Parabasalia	Spirotrichonymphea	Spirotrichonymphida	Holomastigotoididae		
n13	Parabasalia	Trichomonadea	Honigbergiellida	Tricercomitidae	*Tricercomitus*	
n16	Preaxostyla	-	Oxymonadida	-		
n17	Parabasalia	Trychonymphea	Trichonymphida	Trichonymphidae		
n18	Parabasalia					

Table 2: Average number of flagellate protists observed in the haemacytometer area for each termite colony location (Évora, Faial, Leiria and Sesimbra), in Portugal.

	Évora	Faial	Leiria	Sesimbra
n1	5.0	4.7	5.3	5.7
n12	7.0	20.0		6.0
n13		4.2	5.6	4.7
n17		4.0		4.5
n18	5.0	4.9		4.7
n4		6.1	4.0	4.1
n6		4.4	5.4	4.4
n8	23.8	12.3	17.3	16.6
n9	12.2	7.1	4.6	6.0
n16	4.6	4.0	4.0	4.0
n2	16.1	12.0	76.0	26.1
n3	19.0	15.7	16.2	18.6
n5		4.0	4.9	4.3
n7	4.6	5.6	4.0	4.7

Despite this result, the differences between termite colonies of different locations may not be denied, but are supposed to be minor adaptations to their conditions. It is important to note that two populations were collected in natural fields (forests of pines – Sesimbra and Leiria), while the other two populations were collected in urban environments (backyards – Faial and Évora). However knowing the diet of the termites at the time of the collection is not possible, as termite colonies usually rely on different sources of cellulose and workers feeding on different diets may transfer their gut contents to other termites, which were not in direct contact with the food source, through trophallaxis.

3.2. Dietary variation

Termites submitted to different diets were collected from the same colony, and the results shown should clarify if the null hypothesis (different diets do not affect significantly the diversity and abundance of the flagellate protists inside the termites) is to be accepted. Termites were identified to morphotypes (see Table 1) and quantified in terms of abundances (Table 3).

Table 3: Average number of flagellate protists observed in the haemacytometer area for each different diet, after the end of the trials.

	Natural diet	Pine	Beech	Beech TMT	Cellulose	Starving
n1	5.1	6.0	4.1	4.4	5.4	4.0
n12	8.0		5.6	4.0		
n13	4.4	4.5	4.7	4.0	4.7	4.3
n16	4.0	4.7	4.0			5.0
n17		4.0	4.0			
n18	4.0	4.0	5.3	4.0	4.0	
n2	17.0	12.5	19.2	18.7	8.4	9.8
n3	42.0	37.5	32.1	28.3	15.8	17.6
n4	4.9	5.0	4.9	4.4		4.3
n5	5.1	5.1	4.0		4.0	4.0
n6	6.2	4.2	4.0	4.0	4.0	4.0
n7	4.0	5.5	4.9	4.0	4.8	4.0
n8	17.7	20.2	21.9	19.6	23.5	12.9
n9	8.9	8.4	11.6	7.7	4.8	5.2

A paired T-test was performed between the termites submitted to different diets in order to investigate the veracity of the null hypothesis (flagellate protists diversity and abundance is not significantly different between termites submitted to different diets; $p<0.05$) (Table 4). The natural diet fed termites showed no significant differences relatively to termites fed with pine ($t=0.907$; $p=0.191$) and beech ($t=0$; $p=0.499$); and were significantly different from termites fed with beech TMT ($t=1.847$; $p=0.045$), cellulose ($t=1.882$; $p=0.042$) and starving termites ($t=2.277$; $p=0.021$). The termites fed with pine showed no significant differences relatively to the untreated ($t=-0.952$; $p=0.180$) and beech TMT ($t=1.182$; $p=0.130$) and significant differences relatively to termites fed with cellulose ($t=1.903$; $p=0.041$) and starving termites ($t=2.278$; $p=0.021$). Termites fed with beech showed significant differences relatively to termites fed with beech TMT ($t=4.729$; $p<0.001$), cellulose ($t=2.846$; $p=0.007$) and starving termites ($t=3.242$; $p=0.004$). Termites fed with beech TMT were not significantly different from termites fed with cellulose ($t=1.402$; $p=0.093$) or starving termites ($t=1.648$; $p=0.063$). Starving termites and

cellulose fed termites were also no significantly different from each other according with this test (t=0.196; p=0.423).
According with the paired T-test, the non-treated sound woods originated similar flagellate protists communities, while cellulose fed termites and starving termites were significantly different from all woods, except the beech TMT.
The results of the average survival rate of termites submitted to the trials and the average mass loss of the different diets offered to the termites show that, as expected, termites thriving on the natural diet showed a higher survival rate when comparing with the other diets (Table 4).

Table 4: Average survival rate (%) of termites and average mass loss (%) of lignocellulosic substrates offered to termites in the end of the trials.

	Survival rate (%)	Mass loss (%)
Natural diet	74.4	5.2
Pine	39.3	3.7
Beech	45.9	3.6
Beech TMT	39.5	3.7
Cellulose	39.6	11.4
Starving	33.2	-

Starving termites obviously exhibited the more depleted flagellate protist fauna in terms of abundance, since no source of energy was available neither for termites and protists. This method was already applied by other authors for the termite hindgut defaunation (*e.g.* Nunes and Dickinson 1996, Belitz and Waller 1998, Hu *et al.* 2011). The results obtained showed however a good resilience of the flagellate protists community to starvation, and also a certain resilience of the termites, which were still alive (33.2%) after six days of starvation. These facts may be explained whether by the fact that flagellate protists may fed on the bacteria living inside the termite hindgut, or eventually the practice of cannibalism to other protist species, or may be related to the subterranean termite cannibalism/necrophagy habits. Termites practice cannibalism due to scarcity of food or to starvation, although the mechanisms that trigger this behaviour remain obscure, authors hypothesize that the degree of relatedness of termite workers is low (decreasing the altruistic behaviour when under stressful situations), others defend that it is a natural response to the survival struggle, as cannibalism may provide both an additional source of nitrogen and also provide symbiotic fauna in order to maintain the digestion abilities when food becomes available (Hu *et al.* 2011). Necrophagy is a normal behaviour within termite colonies, as it represents an additional income of nitrogen to the termites. However, subterranean termites practice necrophagy only with dead colony members, avoiding actively the dead corpses of termites belonging to different termite species (Sun *et al*. 2013). This fact may be regarded, not only as a strategy of nutrient recycling and defence, but also as a mechanism to maintain the symbiotic fauna communities' integrity (Sun *et al*. 2013).
Cellulose is an energy source to the termites, although much simpler than sound wood, and presumably it requires a less complex digestive process to obtain energy. The higher mass loss shown by cellulose (Table 5) may also be indicative of being a poorer lignocellulosic source. The flagellate protists have different roles in the lignocellulosic digestion process, therefore, the simplification of the digestion process may originate the depletion of the flagellate protists communities, as shown by the data obtained: four morphotypes were absent from the termites fed with cellulose, and also the abundances of some morphotypes (n2, n3, n9, for example) were lower in termites fed with cellulose. However, it was verified that n8 morphotype showed a higher average abundance relatively to the other diets, maybe because this flagellate protist had a

main role in the cellulose degradation. One study stated that host termites contribute more actively to lignocellulose degradation, whereas symbiotic protists contribute more to a pure cellulose diet degradation, as it was a metatranscriptomic study, it is not possible to establish parallel conclusions with the current work (Raychoudhury *et al.* 2013).
Interestingly, the flagellate protists community of untreated beech and beech TMT fed termites were considered to be significantly different, although the heat treatment was not very long, the effects in terms of the lignocellulosic components available to the termites seem to have been significant. It is known that for beech, the thermal treatment leads to a degree of decomposition of wood components, variable with the type of heat treatment applied (Windeisen *et al.* 2007). The common effects of thermal treatment already observed in beech were: loss of functional groups (carboxyl, hydroxyl, and acetyl), an increase of carbon content, solubilisation of lignin, decrease in the pH and the decomposition of sugars (Windeisen *et al.* 2007). All these effects also affect the lignocellulose digestion process; not only directly because of the decrease of possible sources of energy available, but also the associated pH modification may interfere with the hindgut chemical stability.

4. CONCLUSIONS

The results obtained reinforce that termites from the same colony, when submitted to different diets, have the ability to adapt their symbiotic flagellate community in order to efficiently digest the lignocellulosic source available. The cooperation between termite host and its symbiotic flagellate protists microbiota increases their ability to adapt more rapidly to changing conditions (Rosenberg and Zilber-Rosenberg 2013).
The termite symbiotic flagellate protists community is a dynamic assemblage able to adapt to different conditions and diets, although the effects of geographical location were not considered significant, the laboratory diets caused major adaptations of the flagellate protists communities. This fact should be further investigated, using morphological and molecular data, since it may add important information to innovative termite control strategies which are being developed worldwide (*e.g.* Scharf *et al.* 2011, Sethi *et al.* 2014).

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