Insectes Sociaux

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Evidence from Mid-Infrared Spectroscopy (MIRS) that the biochemical fingerprints of *Odontotermes obesus* colonies change according to their geographical location and age

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Abstract In this study, we used Mid-Infrared Spectroscopy (MIRS) to analyze the biochemical fingerprints of nine termite colonies (soldiers, minor and major workers). We then examined whether these spectra could be used to differentiate termite colonies and if their differences could be explained by the geographical distances between colonies and/or their ages. We demonstrated that when only data from the heads of minor workers were considered, the specific fingerprint of each colony appeared to reflect its coordinate position in the study site. However, termite mound height, a proxy for colony age, was the main factor governing these signatures when data from the abdomen of major workers were used. Thus, this study shows the potential of MIRS for differentiating termite colonies in the field. It also highlights the close relationship between the physiological states of termite colonies and their environment.

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Introduction

In tropical ecosystems, termites undoubtedly play an important role in influencing several key ecological functions such as those regulating soil dynamics, nutrient cycling and litter decomposition (e.g., Black and Okwakol 1997; Holt and Lepage 2000). Their ecological success relies on their ability to consume ligno-cellulosic substrates, produce soil biogenic structures (e.g., mounds and sheetings) and their complex social organization. Indeed, termites are eusocial insects and their colonies are composed of individuals divided into well-differentiated castes where each group has specific tasks (workers search for food, build or repair the termitaria, care for eggs and siblings, etc. while soldiers are devoted to protecting the colony) (e.g., Grassé and Noirot 1947; Badertscher et al. 1983; Thorne 1997; Roisin 2000; Ajayi 2012; Chouvenc et al. 2015). The role of polyethism, or division of labor, has been reported for several termite species, including fungus-growing termite species, where physiological differences in gut contents and labial gland depend on the age of individuals and are task-related (Hinze et al. 2002). For instance, Badertscher et al. (1983) suggested that a widespread rule in social insects is that young major and minor workers share the interior tasks while the older workers perform tasks outside the nest. Although soldiers and workers share numerous activities, including fluid transfer via trophallaxis (Spragg and Paton 1980; Cabrera and Rust 1999; Suarez and Thorne 2000; Machida et al. 2001; Nalepa 2015), differences in the nutritional physiology and chemical content (e.g. protein digestibility, tannin content) among termite castes have also been shown (Roisin 2000; Suarez and Thorne 2000; Ajayi 2012). Consequently, it is commonly assumed that the biochemical and organizational properties of termite colonies evolve with time, among castes and are influenced by the environment (Luykx 1986;

Darlington and Dransfield 1987; Waller and La Fage 1988; Hinze and Leuthold 1999; Liu et al. 2005a; Chouvenc and Su 2014).

Infrared spectroscopy is rapidly gaining popularity among biologists for estimating soil properties (e.g., Rossel et al. 2006; Reeves et al. 2010; Cécillon et al. 2008; Gobrecht et al. 2014; Conforti et al. 2015; Clairotte et al. 2016; Madhavan et al. 2016), determining the origin of soil biogenic aggregates produced by termites and earthworms (Hedde et al. 2005; Velasquez et al. 2007; Jouquet et al. 2010; Bottinelli et al. 2013) or characterizing soil nematode communities and differentiating termite species (Barthes et al. 2011; Jouquet et al. 2014) based on their specific biochemical fingerprints. The success of this approach relies in its fast, easy and low-cost implementation and because each sample is assigned to a specific chemical fingerprint. In this study, we examined whether mid-infrared reflectance spectroscopy (MIRS) could also be used to differentiate termite colonies from the same species, and thus constitute a relevant alternative to the traditional approaches which rely on the costly and complex study of agonistic behavior, cuticular hydrocarbon profiles, or the specific genetic fingerprints of termite colonies (Thorne and Haverty 1991; Haverty et al. 1999; Bulmer et al. 2001; Chouvenc and Su 2017). Our hypothesis was that the biochemistry of soldiers and workers' bodies (head versus abdomen) differently reflect the ecology of termite colonies such as their age and/or the environment where they live.

Materials and methods

Study site and model

Samples were collected in the Mule Hole experimental watershed (4.1 km^2). This dry deciduous forest is located in the Bandipur Tiger reserve in southern India at 11°44′N and 76°26′E (Karnataka state, Chamarajanagar district). The Mule Hole watershed is located in the sub-humid zone of the sharp climatic gradient induced by the Western Ghats. As a result of the short-term variability of the southwest monsoon, the experimental watershed is characterized by rainfall ranging from 700 to 1500 mm year^{-1}, with an average over the last 30 years of 1100 mm year^{-1}. The elevation of the watershed ranges from 820 to 910 m a.s.l. The relief is mostly undulating with gentle slopes. The soil is mainly composed of well-drained Ferralsols and impervious Vertisols (Barbiero et al. 2007; Braun et al. 2009). In this environment, the fungus growing termite species *Odontotermes obesus* (Isoptera, Macrotermitinae) builds abundant and monocalic (i.e. one nest per colony) cathedral mound nests with several ridges and conical turrets that are very similar in their shapes to those made by *Macrotermes*

bellicosus in Africa (Roonwal 1970, 1978; Jouquet et al. 2015, 2016). Nine termite mounds were randomly selected in the study site. Their height and GPS coordinates were recorded (Fig. 1).

Termite sampling and MIRS analyses

Two turrets in each mound were broken to stimulate termite building activity. Termites were then collected after 5, 10 and 15 min and preserved in 80% alcohol. In total, approximately 800 individuals were sampled per colony ($n = 9$). They were later sorted into soldiers, major and minor workers in the laboratory and the proportion of each category was counted. For each group, half of the individuals were kept intact and half were separated into head or abdomen samples. Termite individuals or their head or abdomen were dried at 30 °C for two weeks and then were manually ground to a powder < 1 mm. Individuals weighed 0.40, 0.26 and 0.52 mg on average, and the head of individuals represented 37, 28 and 46% of the body mass, for soldiers, minor and major workers, respectively. Composite samples from multiple individuals or their head or abdomen ($w \sim 25$–50 mg) were scanned with a spectrometer (FTIR 660, Agilent ex-Varian) in the mid-infrared (MIR) spectral range from 400 to 4000 cm^{-1} with a KBr separator and a Silicon detector. The reflectance measurements were made at 2 cm^{-1} intervals but a 20 cm^{-1} interval was considered in the analysis. Diffuse reflectance (DR) was transformed to the second derivative according to general procedures recommended to remove baseline shifts and separate overlapping absorption (Reeves et al. 2002).

Statistical analyses

Principal component analyses (PCA) were carried out to compare colonies based on the spectral profiles of their soldiers, minor and major workers [$n = 9$ colonies × 3 categories (soldiers, minor and major workers)]. Monte-Carlo permutation tests were used to assess the statistical significance of differences between colonies from their PCA scores with 999 simulations. ANOVA were then carried out
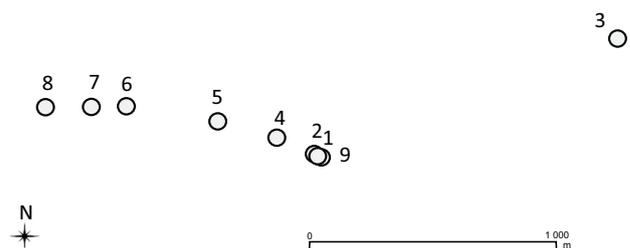

Fig. 1 Distribution of the termite mounds (colonies 1–9) in the study site

to determine significant differences between colonies based on their projections on the first axis of the PCA (since this axis explained most of the variation).

The influence of the environment on the spectral signature of colonies was then tested by comparing the Euclidean distances between colonies from the PCA to their geographical distances using a Mantel test. Because the precision of the GPS was approximately 7 m, the Euclidean distances were averaged for geographical distances < 10 m. A regression model was then carried out to assess significant relationships between Euclidean distances in the PCA and geographical distances, with $n = 16$.

Relationships between termite mound height and MIRS variables were tested using Partial Least Squares Regressions (PLSR, Mevik and Wehrens 2007). PLSR were used to fit linear regression models by projecting predicted and observable variables from the MIRS data as a function of the termite mound height. The most important MIR wavelengths were determined using the variable importance on the projection (VIP) method, which computes scores to each wavelength (Tenenhaus 1998; Cécillon et al. 2008). Models were then tuned to minimize the Root-Mean-Square Error of Cross-Validation (RMSECV) and to maximize the Q^2 value (cross-validated R^2, which gives the predicting ability of the model). VIP wavelengths ($n = 5$) were then used to carry out another PCA comparing colonies based on the spectral profiles of their soldiers, minor or major workers ($n = 9$ samples in all cases). For the best PLSR model, a linear regression was finally used to assess the relationships between the coordinates of the samples on the first axis of the PCA and the height of their termite mounds.

All statistics were calculated using R studio and R version 3.2.1. (R Development Core Team 2012) with the ade4, pls and mdatools packages for PCA, PLSR, Mantel and VIP test analyses, respectively. Differences were declared significant at the 0.05 probability level.

Results

Differentiation of termite colonies

A large number of the individuals collected were soldiers (45.9%, Standard Error SE = 4.5) or minor workers (42.9%, SE = 5.0), and only a small proportion were major workers (11.2%, SE = 2.2). The proportion of soldiers, minor and major workers in each mound was constant across the study site and termite mound height did not influence community structure ($P > 0.05$ in all cases, data not shown).

PCA of the spectral signatures of the nine colonies were carried out on the whole body, or the head and abdomen only (Fig. 2a, b, c). Samples (soldiers, minor and major workers) were distributed along the first two axes which on average
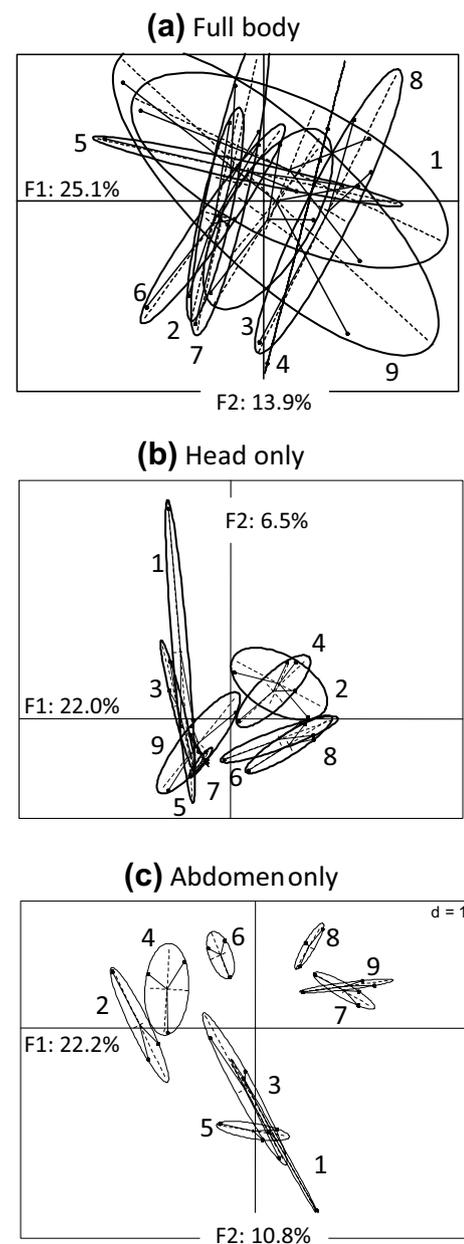

Fig. 2 Projections of the samples (soldiers, minor and major workers from the nine colonies) on the first (F1) and second (F2) principal components showing their explained variance when termites were considered as a whole (**a**) or their head (**b**) or abdomen (**c**) analyzed separately

only explained ~ 30% of the total variability (~ 20% for the first axis and ~ 10% for the second). Individual colonies could not be differentiated according to MIR fingerprints of whole bodies ($P > 0.05$). In contrast, some colonies were separated mainly along the first axis of the PCA when analyses were carried out on the head or abdomen data, with less overlap when abdomen data were considered alone (ANOVA models from the projection of the samples on the first axis,

Table 1 Results of the Mantel test measuring correlations (r and P) between the geographical distance matrix from the nine termite colonies (in m, see Fig. 1) and the Euclidean distance matrix obtained from the first and second axes of the PCA representing the spectral fingerprints of soldiers, major and minor workers from the nine colonies (see Fig. 2)

	Full body	head	Abdomen
Soldiers	$r=-0.32, P=0.983$	$r=0.01, P=0.324$	$r=-0.12, P=0.711$
Minor workers	$r=-0.16, P=0.870$	$r=\mathbf{0.30}, P=\mathbf{0.032}$	$r=-0.11, P=0.764$
Major workers	$r=-0.01, P=0.508$	$r=-0.06, P=0.693$	$r=-0.26, P=0.958$

The whole body, the head or the abdomen of soldiers, minor or major workers were considered separately (n = 9 in all cases). Significant results are highlighted in bold
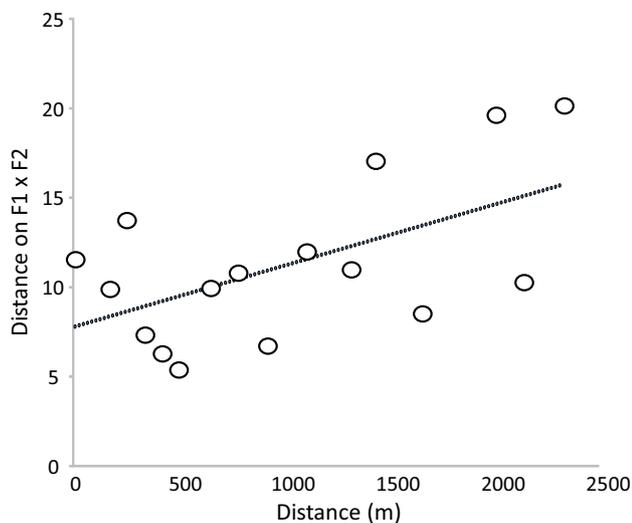

Fig. 3 Relationship between the geographical distance matrix between colonies and the Euclidean distance matrix of their minor worker heads from the PCA shown in Fig. 2. The regression line is represented, $n=16$

$P<0.001$ for both the head and the abdomen). Specific MIR signatures were found for most of the colonies when abdomen data were used, with the exception of colonies 1, 3 and 5 on the one hand and colonies 9 and 7 on the other hand.

Influence of the distance between colonies

The observed overlaps in MIR fingerprints for colonies, as shown in Fig. 2b, c, could not be explained by their geographical proximity (Mantel test, $P>0.05$), except when the spectral signatures of minor workers' heads were considered ($P<0.05$, Table 1). Indeed, a linear regression model was measured for the geographical distance matrix between colonies and the Euclidean distance matrix of their minor workers' heads ($y=0.034x+7.76$, $R^2=0.32$, $P=0.021$, Fig. 3).

Relationship between termite colony and mound height

With a RMSECV between 30 and 45 and Q^2 from -0.94 to 0.06, the worst PLSR models (lowest R^2 and highest RMSE

Table 2 Main results of the best PLSR models testing relationships between the measured and predicted termite mound heights from the spectral signatures of soldiers, minor workers or major workers ($n=9$) and after selection of the five most important variables on the projections (VIP)

	Full body	Head	Abdomen
Soldiers	$R^2=0.53$ RMSE$=22.10$	$R^2=0.06$ RMSE$=31.21$	$R^2=0.42$ RMSE$=23.51$
Minor workers	$R^2=0.29$ RMSE$=27.29$	$R^2=0.48$ RMSE$=22.94$	$R^2=0.55$ RMSE$=21.31$
Major workers	$R^2=-0.45$ RMSE$=38.96$	$R^2=-0.94$ RMSE$=44.82$	$R^2=0.79$ RMSE$=14.81$

R^2 coefficient of determination, *RMSE* root mean squared error

values) were obtained with soldier and major worker heads and with the whole body of major workers (Table 2). Intermediate models were obtained with the other treatments with a Q^2 from 0.29 to 0.55 and RMSECV ~ 25. The best PLSR model was measured with major worker abdomens where the RMSECV and Q^2 reached 14.81 and 0.79, respectively (Fig. 4). The VIP wavelengths used for the best PLSR model were from the MIR regions at 960, 1540, 1680, 1700 and 1720 cm^{-1}. Those VIP wavelengths were then used to carry out a PCA. Coordinates of colonies on the first axis of this PCA, which explained 87.2% of the total variability, were then linearly related to mound height (h (cm) $= 42.45$ $x+95.18$, $R^2=0.46$, $P=0.046$, Fig. 5).

Discussion

The recent and growing literature on the application of infrared spectroscopy in ecology has shown that this technique is useful for differentiating species (Jouquet et al. 2014) and the specific soil properties of soil biogenic structures (Hedde et al. 2005; Velasquez et al. 2007; Cécillon et al. 2008; Jouquet et al. 2009, 2010; Bottinelli et al. 2013). In this study, we examined whether this approach could also be useful for differentiating colonies from the same species, namely, *O. obesus*.
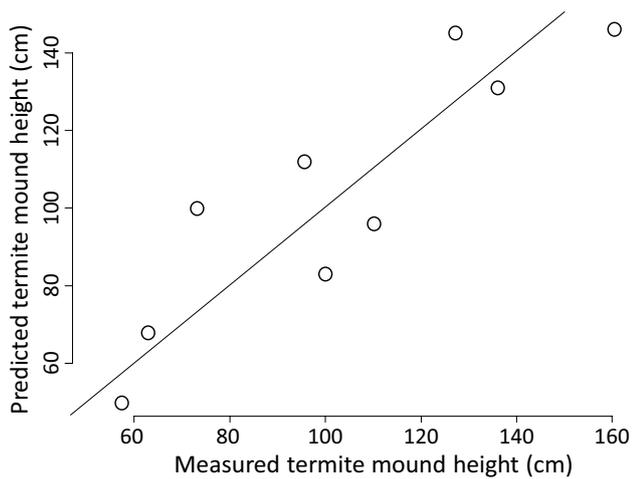
Fig. 4 Results of the PLSR model showing scatter plots of predicted versus measured values for termite mound height (in cm) based on the MIR fingerprints of major worker abdomens and after the selection of VIP wavelengths
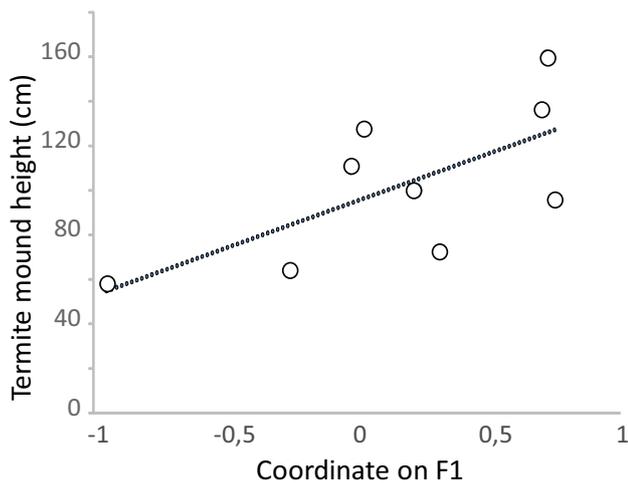

Fig. 5 Relationship between termite mound height (in cm, $n=9$) as a function of the coordinate of samples (major worker abdomens) on the first axis of a PCA carried out from their MIRS signatures and after the selection of the VIP wavelengths

Termite castes

Termites are eusocial insects and their colonies include soldiers and workers, which each play specific roles. Most termite colonies include specific proportions of soldiers and workers (Haverty 1977; Haverty and Howard 1981), although these proportions can change according to the size and age of the colonies (Badertscher et al. 1983; Luykx 1986; Chouvenc and Su 2014) or be influenced by the environment (Waller and La Fage 1988; Liu et al. 2005a, b). In our study, only individuals that were actively contributing

to repair the broken parts of the mounds were sampled. The proportions of soldiers and major or minor workers were constant regardless of termite mound height, used here as a proxy for colony age (Darlington and Dransfield 1987).

Differentiating termite colonies with their specific spectral fingerprints

In agreement with our hypothesis, termite colonies displayed specific fingerprints, which were mainly observed when termite abdomens were analyzed separately from heads. Results of the PLSR models showed that mound height did affect the specific MIR fingerprints of termite colonies, especially when major worker abdomens were analyzed. The VIP method stressed the importance of the wavelengths from 1540 to 1720 cm^{-1} for improving the PLSR model. These wavelengths are mainly attributable to the double-bond region with carbonyl (C=O) and aromatic alkene (C=C) stretching (Stuart 2004; Madari et al. 2006). The PLSR model of major worker abdomen data was also performed with wavelength values at 960 cm^{-1}, which is characteristic of the 'fingerprint region' with single bond bending (e.g., C–H, C–C–C) or stretching (e.g., C–C, C–O, C–N, N–O) (Stuart 2004). In our study the origin of the molecules involved in the PLSR model was not determined. However, despite the fact that a very low number of samples was used ($n=9$ colonies), our model made accurate predictions of termite mound height and a significant linear regression was measured when termite mound height was plotted as a function of the first PCA axis obtained from the spectral signatures of major worker abdomens. As observed in Africa and the Amazon, termite mound size and properties can be considered as a proxy for termite colony size and age (Darlington and Dransfield 1987; Josens and Soki 2010; Pequeno et al. 2013). Although this relationship has never been shown for *O. obesus*, our results suggest that the chemical fingerprints of colonies are not constant but evolve with the aging of the colonies and their exo-symbiotic fungus, together with the growth of their mound nests. However, we cannot discard the hypothesis that the MIR fingerprints of termite colonies also reflect their food resources, and that termites adapt the size of their mounds correspondingly (e.g. for controlling humidity and temperature) (Turner 2004). This hypothesis is in agreement with Nutting (1969) who showed that termite colonies can follow their own timetables for development and reproductive cycles. It is also consistent with Li et al. (2015) who showed that the biochemical composition of insects is highly variable and reflects their developmental stage and diet. Obviously, these hypotheses do not contradict each other and they rather confirm the close relationship between the ecology of termites and their specific biochemical signatures.

The spectral signatures of minor worker heads were also useful for differentiating colonies based on their geographical distances. This result suggests that the environmental properties (e.g., microclimate and type and availability of food) and possibly intra- and inter-specific competition for food and space (Korb and Linsenmair 2001; Davies et al. 2003) also influence the biochemical fingerprints of termite colonies. Interestingly, this relationship was only measured when the data for the heads of minor workers were used in the analysis. Thus this finding suggests that this part of the body better reflects the influence of the environment than the abdomen.

Conclusion

This study highlights the interest of using MIRS for differentiating termite colonies. The identification of termite biochemical fingerprints is undoubtedly easier, faster and cheaper than using DNA to study termite populations (Attignon et al. 2005). This finding is important because it offers a new perspective for studying the ecology of termites and potentially other social insects.

In our experiment, analyses were carried out on a large number of samples (> 30 individuals per MIRS analysis), due to the low weight of individual termites. Therefore, a future perspective of this study would be to determine if the MIRS signature of individual termites can reliably be assigned to those of colonies. If so, this method would be useful for identifying the origin of termites that are observed foraging outside of nests, and especially those from colonies that are close to one another.

Another important conclusion of this study is that it provides further evidence for a link between the physiological states of termite populations, their environment and their mound properties (their height in this case). As outlined by Pequeno et al. (2013), the termite colony-nest system can be seen as a cohesive phenotype at the core of termite ecology and evolution. Further studies are now needed to understand the physiological mechanisms behind the models used in this study. We are especially interested in examining why models of data from minor worker heads appear to better reflect the termite colony environment whereas models of abdomen data from major workers better reflects their age.

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