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Short Communication

# Molecular and biochemical markers for monitoring dynamic shifts of cellulolytic protozoa in *Reticulitermes flavipes*

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**Abstract**

Lower termites rely on cellulolytic protozoa to aid in the digestion of their wood-based diet. However, despite the major contribution of protozoa to the lower termite digestive system, few techniques have been developed to monitor shifts in protozoan populations. This study investigated whether quantitative real-time PCR (qRT-PCR) and/or cellulase enzyme assays can be used to monitor changes of cellulolytic protozoan populations in the lower termite, *Reticulitermes flavipes* (Kollar). Previously developed cellulase primer sets were used to test for changes in cellulase gene expression, while three different cellulase enzyme assays were used to assess changes in cellulase enzyme activity. The results from this study indicate that qRT-PCR is a reliable method to monitor shifts in cellulolytic protozoan populations. Specifically, qRT-PCR can serve as a useful monitoring technique during high-throughput screening of novel termite control agents such as cellulase inhibitors, and help to answer questions relating to whether or not such control agents impact cellulolytic protozoan populations.
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## 1. Introduction

The mutualism between lower termite species and a variety of symbiotic protozoa has been of interest to termite researchers since early studies done by Cleveland (1924). Symbiotic protozoa have long been known to be important to the process of termite cellulose digestion. Namely, protozoan species produce cellulases, which are enzymes capable of cleaving the 1,4-$\beta$-D-glucosidic linkages in cellulose (a major component of wood). In recent years, additional advances have been made in characterizing the cellulase system of many lower termite species including *Reticulitermes flavipes* (Kollar), *R. speratus* (Kolbe) and *Coptotermes formosanus* (Shiraki) (Inoue et al., 1997; Nakashima et al., 2002; Zhou et al., 2008).

The cellulase system in lower termites is known to involve both endogenous and symbiotic cellulases (Watanabe et al., 1998; Nakashima et al., 2002; Tokuda et al., 2005; Zhou et al., 2008). Unlike symbiotic cellulases which are confined to the hindgut, endogenous cellulases are produced and found in the salivary glands or the midgut, depending on termite family (Tokuda et al., 1999; Zhou et al., 2007). This study investigated the cellulase system of the lower termite *R. flavipes*, which is one of the most destructive termite species in the United States. The *R. flavipes* cellulase system, like that of all other lower termite species, is known to comprise three main types of enzymes including endoglucanases, exoglucanases, and $\beta$-glucosidases. These enzymes degrade cellulose by cleaving different linkages along the cellulose chain. Endoglucanases internally hydrolyze glucosidic linkages while exoglucanases terminally cleave cellulose units from the ends of cellulose chains. $\beta$-glucosidases release glucose by hydrolyzing $\beta$-D-glucose residues from cellobiose and cellotriose units (Breznak and Brune, 1994).

Thus far, the full-length protein coding regions of four cellulase genes have been isolated from *R. flavipes*,

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including an endogenous (*Cell-1*) and three symbiotic cellulases (*Cell-2*, *Cell-3*, and *Cell-4*). The endogenous *Cell-1* and the symbiotic *Cell-2* are classified as endoglucanases, while *Cell-3* and *Cell-4* are exoglucanases. In *R. flavipes*, endogenous endoglucanase activity is mainly localized in the salivary glands and symbiotic cellulase activity is localized in the hindgut (Zhou et al., 2007).

The process of cellulose degradation can sustain nearly 100% of lower termite metabolism (Breznak and Brune, 1994). Thus, given the dependence of lower termites on wood, the lower termite cellulase system is considered a potential target for termite control (Zhu et al., 2005, 2008). However, despite the major role of protozoa in the lower termite cellulase system, to date there are few techniques beyond actual protozoan counts available to monitor the effects that novel termite control agents could have on cellulolytic protozoan populations. Although protozoan counts provide a quantitative and direct measure of protozoan populations, they are also time consuming and labor intensive. Therefore, the main goal of this investigation was to develop an effective, alternative method to monitor fluctuations of cellulolytic termite protozoa. Specifically, the objectives of this study were: (1) to use UV irradiation to remove hindgut protozoa from *R. flavipes* workers and (2) to test whether quantitative real-time PCR (qRT-PCR) and/or cellulase enzyme assays can be used to monitor changes in termite cellulolytic protozoan populations. In addition, an estimate of endoglucanase, exoglucanase, and β-glucosidase stability *in vitro* was also included. Our results suggest that qRT-PCR is a viable method to monitor shifts in cellulolytic protozoa populations and verify specifically that the *R. flavipes* genes *Cell-2*, *Cell-3*, and *Cell-4* are symbiont-derived.

## 2. Materials and methods

### 2.1. Termites

Termite colonies were collected from the University of Florida campus (Gainesville, FL, USA). Each colony was placed in a sealed plastic container and supplied with moist, brown paper towels and wooden shims (pine) as food. The termite colonies were held in complete darkness at approximately 22 °C and 70% RH for at least a month before including them in these experiments.

### 2.2. Ultraviolet irradiation

UV irradiation was successfully used as a defaunation method in *R. speratus* by Inoue et al. (1997). Their irradiation protocol was applied to this study with minor modifications. Prior to UV irradiation, termites were fed moistened cellulose powder (Sigma-Aldrich) for 48 h, in order to make hindgut contents more accessible to UV light. Thirty worker termites were irradiated (375.5 nm, Gelman Sciences Inc., Model No. 51438) for 0, 1, 2, 3, or 4 h. The UV light was placed at a distance of approximately 9 cm away from the dishes. In order to ensure that termites would be evenly exposed to the UV light, the 30 termites were divided into three petri dishes (10 × 15 mm, Nunc) and were lined up under the UV light, exposed, then transferred to petri dishes provisioned with moistened filter paper disks for 24 h. After the 24 h period elapsed, termites that were exposed for the same time interval were recombined ($n = 30$). Five termites were used immediately for protozoan counts, while the remaining 25 were divided roughly in half and stored at either −80 °C for qRT-PCR analysis or at −20 °C for cellulase enzyme assays. Treatments were replicated across three different *R. flavipes* colonies.

### 2.3. Protozoan counts

In order to estimate the number of protozoa in each termite hindgut, a total of five termites for each time interval were dissected per replicate. Dissections were carried out by immobilizing each termite on ice, gently holding the head and then extracting the hindgut by pulling on the last abdominal segments. The protocol used for protozoan counts was described by Lewis and Forschler (2004) with minor modifications. Protozoan counts were conducted by placing each hindgut into a microcentrifuge tube containing 100 μl of ice-cold 1 × phosphate buffered saline (PBS). The gut was homogenized for approximately 15 s with a sterile toothpick, then 10 μl of the hindgut homogenate was loaded onto a hemacytometer (Bright-line Hemacytometer, Fisher Scientific, Pittsburgh, PA) and the number of protozoa was examined by counting 0.4 μl under 400 × magnification using a Leitz Laborlux S microscope.

### 2.4. Quantitative real-time PCR

In order to test for changes in cellulase gene expression, total RNA from whole termites was extracted using the SV total RNA isolation kit (Promega, Madison, WI). The quantity of total RNA was determined through spectrophotometry and then converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using an iCycler iQ real-time detection system (Bio-Rad) with SYBR Green® Supermix (Bio-Rad). qRT-PCR was used to investigate gene (mRNA) expression of four cellulase genes (*Cell-1*, *Cell-2*, *Cell-3*, and *Cell-4*), as well as *β-actin* which was included as a reference gene. Validation of these target and reference genes, including comparisons of PCR amplification efficiencies, was described in a previous report (Zhou et al., 2007). Cellulase gene transcript abundance was chosen to specifically monitor changes in cellulolytic protozoan populations, rather than as-yet undefined genomic DNA sequence markers. The forward and reverse primer sequences used were: *Cell-1* RT (5′-TCACAAGCAAGCAGGCATAC-3′ and 5′-ATGAGAGCAGAATTGGCAGC-3′), *Cell-2* RT

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