Identification of the endo- and ectosymbiotic bacteria and cellulolytic activities of the symbiotic flagellates of the Australian termite *Mastotermes darwiniensis*

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Abstract: Identifizierung der endo- und ectosymbiontischen Bakterien und cellulolytischen Aktivitäten der symbiontischen Flagellaten der australischen Termite *Mastotermes darwiniensis*.

Termiten sind mit die wichtigsten holzabbauenden Insekten. Die Darmmikrobiota spielt eine unverzichtbare Rolle im Abbau der Nahrung. Sie besteht aus Bakterien, Archaebakterien (Archaea), Flagellaten und Hefen. Die einzigartigen Flagellaten der Termiten sind sehr früh in der Evolution der Eukaryoten abgezweigte Einzeller, die zu den Preaxostyla (Oxymonadida) und Parabasalia (Cristamonadida, Spirotrichonymphida, Trichomonadida, Trichonymphida) gehören. Die australische Termite *Mastotermes darwiniensis* ist die einzige heute noch lebende Art der primitiven Termitenfamilie Mastotermitidae. In der Gärkammer im Hinterdarm leben die vier größeren Flagellaten *Koruga bonita*, *Deltotrichonympha nana*, *Deltotrichonympha operculata* und *Mixotricha paradoxa*. Weiterhin kommen die zwei kleineren Flagellaten *Metadevescovina extranea* (Cristamonaden) und *Pentatrichomonoides scroa (*Trichomonaden) vor. Die Flagellaten selbst sind Wirte von ecto- und endosymbiontischen Prokaryoten.

Von Zellextrakten der nichtkultivierbaren größeren Flagellaten wurden zwei Endoglucanasen mit einer ähnlichen apparenten Masse von 36 kD isoliert. Sie zeigten signifikante Homologie zu termiteneigenen Cellulasen. Die entsprechenden Gene wurden nicht im mRNA-Pool der Flagellaten gefunden, sondern in den Speicheldrüsen von *Mastotermes darwiniensis*. Das deutet darauf hin, dass die intestinalen Flagellaten auch die Wirtsenzyme für die Cellulosehydrolyse benutzten. Andererseits besitzen mindestens *Koruga*- und *Deltotrichonympha*-Species auch eigene Cellulasegene. Im Darminhalt der Termiten wurden allerdings auch drei Cellulasen nachgewiesen, die von den Flagellaten stammen sollten.

Key words: Termites, *Mastotermes,* Intestinal Microbiota, Flagellates, Cellulases, Spirochetes

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The Australian termite *Mastotermes darwiniensis*

The lower wood-feeding Australian termite *Mastotermes darwiniensis* Froggatt (Fig. 1) is the only living member of the family Mastotermitidae. The complex symbiotic hindgut flora consists of protozoa (formerly named Archaezoa; Cleveland & Grimstone 1964; Brugerolle & al. 1994; Berchtold & König 1995; Fröhlich & König 1999a, b), bacteria (Berchtold & König 1996; Berchtold & al. 1999), archaea (Fröhlich & König 1999a, b) and yeasts (Prillinger & al. 1996; Schäfer & al. 1996).

The digestive system of *Mastotermes darwiniensis* consists of the foregut with the crop and the gizzard, the midgut, and the hindgut (Noirot & Noirot-Timothée 1969; Noirot 1995). The hindgut consists of five segments (P1 – P5): the proctodeal segment, the enteric valve, the paunch, the colon and the rectum. The paunch is the main microbial fermentation chamber, but the colon also contains microorganisms. The paunch is subdivided into a dilated thin-walled region (P3a) and a thick-walled more tubular region (P3b) (Fig. 1c). In the case of *Mastotermes darwiniensis* oxygen diffusion gradients could be detected up to 100 µm below the epithelium (Berchtold & al., 1999).

The symbiotic flagellates

Six species of parabasalid flagellates (cristamonads and trichomonads) inhabit the gut of *Mastotermes darwiniensis* (Table 1; Fig. 1). The flagellates preferentially colonize the P3a region of the paunch (Berchtold & al. 1999). About 95% of this part of the paunch is tightly packed with large flagellates. Approximately 90% of the DAPI-stained bacterial cells were associated with the protozoa in the P3a region, only 2% were attached to the gut wall and the rest was found in the residual liquid volume of the lumen fraction. Besides the larger flagellates the two smaller species *Metadevescovina extranea* and *Pentatrichomonoides scroa* thrive also in the gut of *Mastotermes darwiniensis*.

Table 1. Flagellates living in the gut of *Mastotermes darwiniensis*

Species	Length (µm)	Titer (ml^{-1})
1. Cristamonads (Combined titer of *Deltotrichonympha nana, Deltotrichonympha operculata, Koruga bonita*)	100 – 550	$10^4 – 10^5$
Mixotricha paradoxa	300 – 500	$10^3 – 10^4$
Metadevescovina extranea	15 – 20	ca. 10^7
2. Trichomonads		
Pentatrichomonoides scroa	25	ca. 5×10^6

One of the larger flagellates in the hindgut of *Mastotermes darwiniensis* is *Mixotricha paradoxa*, a member of the Devescovinidae (cristamonads) (Adl & al. 2005) (Fig. 1; Table 1). It ingests wood particles. The surface of *Mixotricha paradoxa* shows a highly ordered pattern of rod shaped bacteria and in addition it is covered by a dense carpet of spirochetes with exception of the posterior ingestive zone (Cleveland & Grimstone 1964; Cleveland 1966c; Cleveland & Cleveland 1966; Wenzel & al. 2003; Brugerolle 2004). The rod shaped bacteria and the spirochetes are attached to regularly arranged protrusions (brackets) of the cell surface. Interestingly, Cleveland & Grimstone (1964) found that the spirochetes and not the relatively small four flagella propel the cells.

Other examples of symbiotic cristamonad flagellates are *Deltotrichonympha operculata*, *Deltotrichonympha nana* and *Koruga bonita* (Fig. 1; Table 1) (Cleveland 1966a, b). The cells of *Deltotrichonympha operculata* and *Deltotrichonympha nana* are characterized by numerous flagella, which cover the anterior part of a cell. At the posterior part the ingestive zone is located. In contrast to *Deltotrichonympha* sp., *Koruga bonita* possesses no dense population of ectosymbiotic spirochetes at the posterior cell part (Fröhlich & König 1999a).

One of the smaller symbiotic trichomonad flagellates is *Pentatrichomonoides scroa* (Fig. 1). This species is characterized by the presence of 5 anterior flagella originating from a groove and an undulating membrane with a recurrent flagellum. The cell body is generally slender with a truncated posterior end and a spiralling undulating membrane around the cell body, but also more massive forms exist (Brugerolle & al. 1994). About 50 methanogens of the genus *Methanobrevibacter* were found as endosymbionts of *Pentatrichomonoides scroa* (Fröhlich & König 1999a). The other smaller flagellate *Metadevescovina extranea* possesses three anterior flagella and a trailing flagellum (Fig. 1).

The phylogenetic tree of parabasalids (Fig. 2; König & al. 2005) shows that four hindgut protozoa (*Metadevescovina extranea*; *Koruga bonita*, *Deltotrichonympha nana*, *Deltotrichonympha operculata*) of *Mastotermes darwiniensis* form one monophyletic subdivision. *Mixotricha paradoxa* exhibits an earlier emergence, indicating its more primitive position. *Koruga bonita*, *Deltotrichonympha nana* and *Deltotrichonympha operculata* are classified in the Deltotrichonymphidae, which indicates that the hypermastigid flagellates are not a monophyletic branch (Fröhlich & König 1999b) and that the hypermastigont system was invented several times (e.g. Deltotrichonymphidae, Trichonymphidae) in the evolution of the flagellates. *Pentatrichomonoides scroa*, the second small intestinal flagellate of *Mastotermes darwiniensis* is not closely related to the other five symbiotic flagellates, because it clusters within the Trichomonadinae.

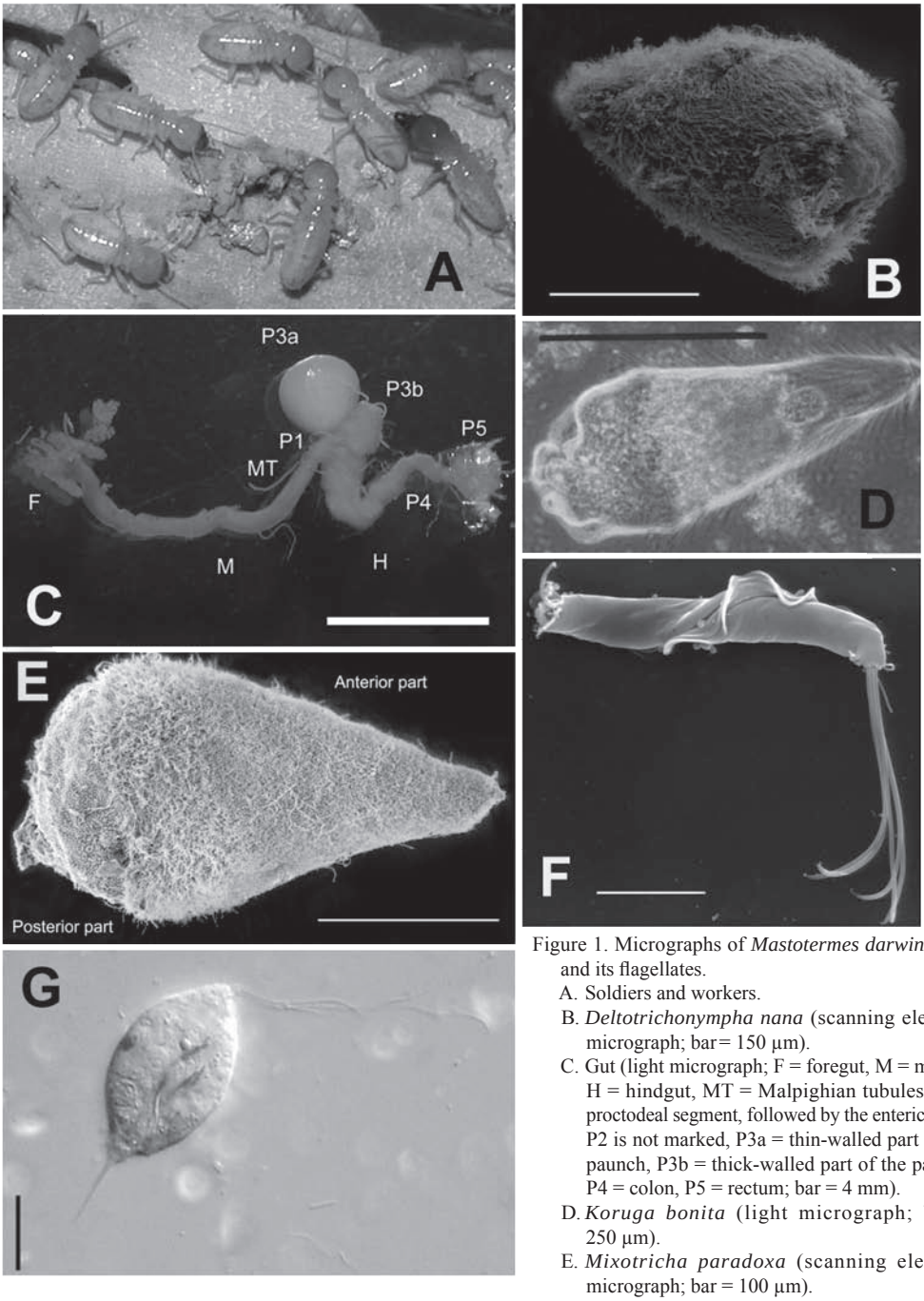

Figure 1. Micrographs of *Mastotermes darwiniensis* and its flagellates.
A. Soldiers and workers.
B. *Deltotrichonympha nana* (scanning electron micrograph; bar = 150 µm).
C. Gut (light micrograph; F = foregut, M = midgut, H = hindgut, MT = Malpighian tubules, P1 = proctodeal segment, followed by the enteric valve, P2 is not marked, P3a = thin-walled part of the paunch, P3b = thick-walled part of the paunch, P4 = colon, P5 = rectum; bar = 4 mm).
D. *Koruga bonita* (light micrograph; bar = 250 µm).
E. *Mixotricha paradoxa* (scanning electron micrograph; bar = 100 µm).
F. *Pentatrichomonoides scroa* (scanning electron micrograph; bar = 5 µm).
G. *Metadevescovina extranea* (light micrograph; bar = 10 µm).

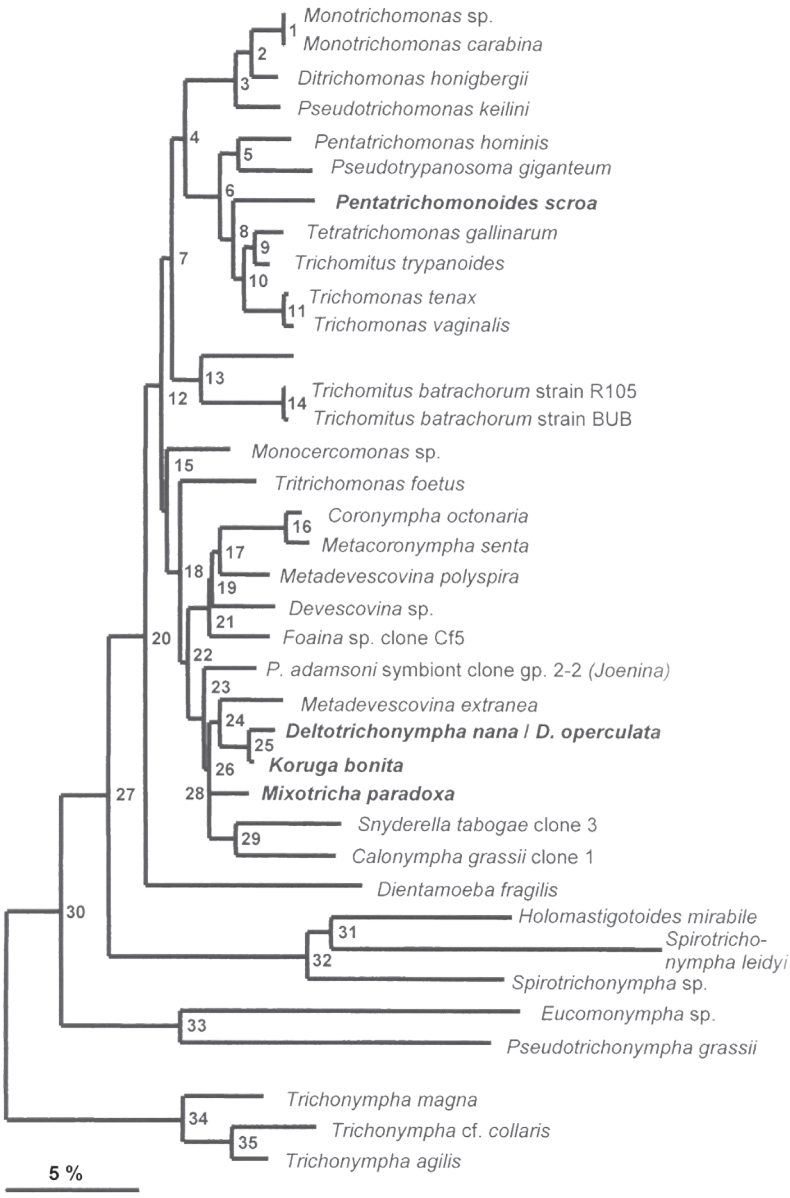

Figure 2. Unrooted phylogenetic tree of the symbiotic parabasalids of *Mastotermes darwiniensis*.
Neighbor-joining analysis of SSU rDNA sequences. Bar represents 5 substitutions per 100 nucleotides. The bootstrap values are computed by three different reconstruction methods: distance matrix, maximum parsimony and maximum likelihood. Asterisks designate nodes with bootstrap values below 40% (LI 2003). Bootstrap values: 1=100/100/100; 2 = 88/97/92; 3 = 100/100/96; 4 = 64/62/50; 5 = 98/97/98; 6 = 100/99/99; 7 = 88/*/43; 8 = 86/80/*; 9= 79/*/*; 10 = 93/50/*; 11 = 100/100/100; 12 = 54/*/*; 13 = 90/100/92; 14 = 100/100/100; 15 = 49/43/*; 16 = 100/100/100; 17 = 76/43/*; 18 = 83/*/*; 19 = 44/67/*; 20 = 72/45/*; 21 = 89/84/56; 22 = 75/45/*; 23 = 59/58/40; 24 = 65/69/63; 25 = 100/100/99; 26 = 42/71/ 58; 27 = 100/84/*; 28 = 100/48/48; 29 = 100/94/95; 30 = 88/98/100; 31 = 92/83/92; 32 = 100/100/100; 33 = 100/100/100; 34 = 100/100/100; 35 = 100/100/99.

Endosymbiotic prokaryotes

The trichomonad *Pentatrichomonoides scroa* (Brugerolle & al. 1994; Berchtold & König 1995), one of the smaller gut flagellates of *Mastotermes darwiniensis,* harbours about 50 endosymbiotic methanogens, which are recognized by their greenish fluorescence under ultraviolet irradiation (Fröhlich & König 1999a). Ten single cells of the endosymbiotic methanogens were isolated by micromanipulation (Fröhlich & König, 1999b). *Methanobrevibacter* sp. (AJ132468) was related to clone CD 3 from the termite *Cryptotermes domesticus* (Ohkuma & Kudo 1998) and *Methanobrevibacter curvatus* from *Reticulitermes flavipes* (Leadbetter & Breznak 1996).

Bacterial cells were aspirated from the cytoplasm of the flagellate *Koruga bonita* (Fig. 1) by using a glass capillary with an opening of 0.5 µm in diameter. The micromanipulated cells *(Mycoplasma* sp.; AJ132469) (Fröhlich & König 1999b) were related to *Mycoplasma alvi* (Pettersson & al. 1996).

Ectosymbiotic prokaryotes

Spirochetes possess a cellular ultrastructure which is unique among eubacteria (Holt 1978; Canale-Parola 1991; Paster & al. 1996). Although spirochetes are always a dominant part of the microflora of all termites (Margulis & Hinkle 1992), only five species have been obtained in pure culture (Leadbetter & al. 1999; Dröge & al. 2006a, b). The so far identified spirochetal clones cluster with the genera *Treponema* (Berchtold & al. 1994; Berchtold & König 1996; Lilburn & al. 1999; Ohkuma & al. 1999) as well as with *Spirochaeta* (Dröge & al. 2006a)

Ectosymbiotic bacteria of flagellates can be detected by electron microscopy (Radek & al. 1992; Dyer & Khalsa 1993; Radek & Tischendorf 1999; Radek & al. 1996; Brugerolle 2004) or after staining the cells with ethidium bromide (Fröhlich & König 1999a). Ectosymbiotic spirochetes have been identified on the surface of flagellates (Iida & al. 2000; Noda & al. 2003; Wenzel & al., 2003). *Mixotricha paradoxa* is a rare example of a movement symbiosis between eukaryotic and prokaryotic microorganisms (Wenzel & al., 2003; Brugerolle, 2004).

Sutherland (1933) published an article about *Mixotricha paradoxa* where the attached spirochetes were misconceived as cilia. A detailed description of the fine structure of *Mixotricha paradoxa* and the role of the ectosymbiotic bacteria in cell locomotion was provided by Cleveland & Grimstone (1964) as well as by Brugerolle (2004).

Cleveland & Grimstone (1964) found two spirochete morphotypes on the surface of *Mixotricha paradoxa*, a small one, which covered the surface of the flagellate as a dense carpet and a longer spirochete, which appeared sporadically and were only loosely bound to the spirochete carpet. Cleveland & Grimstone (1964) described the regular arrangement of the spirochetes and a rod-shaped bacterium attached to the so-called brackets on the cell surface. These brackets seem to be significant for the locomotory function of the spirochetes. They form a regularly posteriorly oriented attachment site for the spirochetes, which allows the spirochetes to propel the flagellate cells forward. The rod shaped-bacteria, which are attached to the anterior site of the brackets, have no part in the locomotion of *Mixotricha paradoxa* (Cleveland & Grimstone, 1964; König & Breunig, 1997).

Wier & al. (2001) described a pillotinaceous spirochete (*Canaleparolina darwiniensis*) from the surface of *Mixotricha paradoxa*. The spirochete occurs also free-swimming in the paunch lumen of *Mastotermes darwiniensis*. This species possesses 16 periplasmic flagella (16:32:16).

Brugerolle (2004) described in addition to the slender spirochetes a larger spirochete with a length of 30 µm and a diameter of 0.5 µm. This spirochete possessed 18 - 30 flagella arranged in two rows which are located in a ridge on the surface. The morphology is similar to that of the described genus *Canaleparolina*. In addition two as yet undescribed rods (length of about 4 µm, diameter of about 0.5 µm) were abundant in the posterior part of the flagellate.

Berchtold & König (1996) found about 13 different spirochetal 16S rDNA clones in the intestinal tract of *Mastotermes darwiniensis*. Spirochetes constitute a main part of the gut flora of these termites (Berchtold & al. 1994; Berchtold & König 1996). Their function in the gut of termites remained unclear for a long time. Today we know that they ferment mono- and oligosaccharides, form acetate from CO_2 and H_2 and fulfil a locomotory function (Cleveland & Grimstone 1964; Cleveland & Cleveland 1966; Leadbetter & al. 1999; Wenzel & al. 2003; Brugerolle 2004; Dröge & al. 2006a, b).

The bacteria associated with the cell surface of *Mixotricha paradoxa* were identified. Six spirochetal 16S rDNA clones (mpsp 15, sp 40-7, mp1, mp3, mp4, mp5) were obtained from the bacteria on the cell envelope of *Mixotricha paradoxa* (Wenzel & al. 2003). Two clones (mpsp15, sp 40-7) were nearly identical (99%) to already described clones (Berchtold & al. 1994; Berchtold & König 1996), while the 16S rDNA sequences of clones mp1, mp3, mp4 and mp5 have not been found previously.

The "spirochete" tree including the 16S rDNA sequences of the six spirochete clones mpsp15, sp 40-7, mp1, mp3, mp4, and mp5 obtained from the ectosymbiotic bacteria together with some representative spirochetes from the EMBL-database (Berchtold & König, 1995) was constructed. The ectosymbiotic spirochetes of *Mixotricha paradoxa* belong all to the *Treponema* branch (Berchtold & al., 1994; Berchtold & König, 1996; Paster & al., 1996; Lilburn & al., 1999; Ohkuma & al., 1999; Iida & al., 2000).

Wenzel & al. (2003) obtained 16S rDNA amplificates (e.g. clone B6; ca. 400 bp) from *Mixotricha paradoxa*, which were related to *Bacteroides forsythus*. Rod shaped bacteria (length: 0.8 - 1.1. µm; width: 0.3 µm) are attached to cell surface brackets in a regular pattern (Cleveland & Grimstone 1964; König & Breunig 1997) perpendicular to the cell of *Mixotricha paradoxa*. The distance between the cells in a row is about 0.9 µm and between two adjacent rows is 0.5 µm. The individual rods in the rows are staggered.

Fluorescence *in situ* hybridization was performed with specific Cy3-labelled probes derived from 16S rDNA sequences obtained from the ectosymbiotic spirochetes and rod-shaped bacterium clone B6. Three spirochetal clones could be localized on the cell surface, clone mpsp15 at the anterior and clones mp1 and mp3 at the posterior part.

The fluorescent probes B6.1 and B6.2 were specific for the *Bacteroides forsythus*-related clone B6. Positive hybridization results showed that clone B6 is spread all over the surface of *Mixotricha paradoxa* in a similar regular pattern as found in electron micrographs.

Glycolytic enzymes of *Mastotermes darwiniensis* and its flagellates

Several glycolytic activities were found in cell extracts of the flagellates Not the total activity of the cellulases seemed to be formed by the flagellates themselves. Two endoglucanases Cel I and Cel II, with the molecular mass of approx. 48 kD, were isolated from cell extracts of the not yet culturable symbiotic hindgut flagellates (Li & al., 2003). The N-terminal sequences of these cellulases exhibited significant homology to cellulases of termite origin, which belong to glycosyl hydrolase family 9. The corresponding genes were not detected in the mRNA pool of the flagellates, but in the salivary glands of *Mastotermes darwiniensis*. A protein with the molecular mass of approx. 48 kD was also detected in crude extract of these flagellates by western blot analysis using a polyclonal antiserum against the cellulase of the termite *Mastotermes darwiniensis*. The results gave evidence that cellulases occurring in the nutritive vacuole of the flagellates partly originated from the termite host. Probably, the cellulases are secreted from the salivary glands of *Mastotermes darwiniensis*. During the mechanical grinding of the wood particles by the termites, the cellulases are attached to wood particles or mixed with them, then the attached cellulases move to the hindgut where they are most probably endocytosed by the flagellates. It has also been found that 40% of the endoglucanase activity of *Mastotermes darwiniensis* is present in the hindgut and most (ca. 84%) of the cellulase activity of the whole hindgut is present in the flagellate extract (Veivers & al. 1982). This implies that a certain amount of termite cellulases, secreted from the salivary glands, moves into the hindgut and enters the flagellate cells. They may be involved in the digestion of cellulose in the flagellate cells.

Using a PCR-based approach DNA encoding cellulases belonging to glycosyl hydrolase family 45 were obtained from micromanipulated nuclei of the flagellates *Koruga bonita*, *Deltotrichonympha nana* and *D. operculata* (Li & al., 2003). The cellulase sequences of the termite symbiotic protists were phylogenetically monophyletic, showing more than 84% amino acid identity with each other. The deduced cellulase sequences of termite origin and flagellate origin consist of a single catalytic domain, lacking a cellulose-binding domain (CBD) and a spacer sequence found in most microbial cellulases.

In the gut extracts of wild termites (*Mastotermes darwiniensis*) a cellulase activity (hydrolase family 45) (Watanabe & al. 2006) which was identical to the amino acid sequence of one mRNA sequence isolated by Li & al. (2003) was found in approximately equal magnitude to termite-derived cellulases, indicating that the flagellates also produce cellulases.

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