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Immunological and ultrastructural characterization of spirotrichonymphid flagellates from *Reticulitermes grassei* and *R. flavipes* (syn. *R. santonensis*), with special reference to *Spirotrichonympha*, *Spironympha* and *Microjoenia*

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Abstract

Five species of spirotrichonymphids representing three genera have been studied by light and immunofluorescence microscopy, and by transmission electron microscopy. The genus *Spirotrichonympha*, represented by *S. flagellata* from *Reticulitermes grassei*, is characterized by a compound axostyle composed of several fibers or subaxostyles. The genus *Spironympha*, represented by *S. kofoidi* from *Reticulitermes flavipes* (syn. *R. santonensis*) and by the two new species *S. verticis* and *S. lanceata*, is characterized by flagellar lines restricted to the anterior area and a simple, tubular axostyle. *Spironympha verticis* and *S. lanceata* are mainly distinguished by ultrastructural details of their flagellar lines and axostyle. These three *Spironympha* species were found in hosts identified as *R. flavipes* or *R. santonensis*, but not in *R. grassei*. This provides additional support for the synonymy of *R. santonensis* with *R. flavipes* recently demonstrated by molecular methods. The generic diagnosis of *Microjoenia* is emended, based on an ultrastructure study of *M. fallax*.

Keywords: Biodiversity; Protozoa; Parabasalia; Termites; Immunofluorescence; Ultrastructure

Introduction

Mapping biodiversity by the identification and description of species is a continuing challenge. This task has not been completed (May 1990), particularly not for microorganisms such as bacteria, archaea (Pace 1997; DeLong and Pace 2001) and protists (Patterson 1999, 2001; Finlay 2001; Dawson and Pace 2002). This is especially true for the symbiotic protozoa living in the hindgut of 'lower termites' that are involved in wood, grass and soil digestion (Inoue et al. 2000; König et al. 2002). These protozoa comprise parabasalid and oxymonad flagellates that were mostly identified by light microscopy in the early 20th century (Grassé 1952; Yamin 1979; Brugerolle and Lee 2001). However, this primary identification of the symbiotic protozoa has not been performed in many termite species (Yamin 1979), or is incomplete or sometimes unusable. Over the last 30 years, electron microscopy has proved an important tool for characterizing the genera (Patterson 1999, 2001; Taylor 1999; Brugerolle and Patterson 2001), but several genera harbored by termites could not be studied by this

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technique (Hollande and Carruette-Valentin 1971; Brugerolle and Lee 2001; Brugerolle and Radek 2006). Molecular identification of the genera and species living in termites is in its early stages (Gunderson et al. 1995; Keeling et al. 1998; Gerbod et al. 2002; Keeling 2002; Moriya et al. 2003; Ohkuma et al. 2000, 2005); many sequences from these works have not been assigned to named taxa. One of the major obstacles to such studies is the lack of primary identification of genera and species by light microscopy, which is necessary to assign a sequence to a species by in situ hybridization. It is also important to point out that termite flagellates often cannot be cultivated but have been successfully isolated with a micromanipulator (Fröhlich and König 1999). Therefore, sequence amplification is performed on isolated organisms, or on the whole content of the termite gut, and the sequences have to be screened and assigned afterwards. Moreover, the symbiotic fauna in a termite is complex; some 'lower termite' species harbor about 10 genera and 20 species of protozoa, which complicates identification by either microscopy or molecular biology.

We have undertaken to better characterize spirotrichonymphids (Brugerolle 2001; Brugerolle and Bordereau 2004) that are symbiotic polymastigote parabasalids largely represented in 'lower termites' (Grassé 1952; Honigberg 1970; Mannesmann 1972; Yamin 1979). In a first study of the *Porotermes* flagellates from Australia, the genera *Microjoenia*, *Spirotrichonympha* and *Spirotrichonymphella* were compared by electron microscopy (Brugerolle 2001). In a second study of *Hodotermopsis sjoestedti* flagellates, the genus *Holomastigotes* was characterized (Brugerolle and Bordereau 2004). The latter study has been completed by the characterization of *Spirotrichonympha*, *Spironympha* and *Microjoenia* species from *Hodotermopsis sjoestedti* (Brugerolle 2005). In the course of these studies, it was discovered that species of these three genera also occur in termites living in Europe, that they had not been studied by electron microscopy, and that some of these spirotrichonymphid species are new to science.

Since the first cytological description of the flagellates of *Reticulitermes lucifugus* by Grassi (1917), many additional studies have contributed to identifying the flagellates of such termites in Europe, as reported by Yamin (1979). One major contribution was that of Duboscq and Grassé (1928) who described *Spirotrichonympha flagellata* from *Reticulitermes grassei* and *Spironympha kofoidi* from *R. santonensis*, a termite first identified by Feytaud in France (Feytaud 1924; Clément 1978; Vieau 2001).

For the present study, we used light-, immunofluorescence and electron microscopy to study the flagellates of termites identified as *Reticulitermes grassei* Clément, 1978, *R. santonensis* (Feytaud, 1924) from France, and as *R. flavipes* (Kollar, 1837), a species reported from the USA (Austin et al. 2002) and also from various areas of Europe (Ye et al. 2004). Our study not only provides new data to characterize the spirotrichonymphid genera *Spirotrichonympha*, *Spironympha* and *Microjoenia*, but also identifies two new *Spironympha* species in *Reticulitermes santonensis* that are also present in *R. flavipes*. This supports the synonymy between *R. flavipes* and *R. santonensis*, which has been long suspected (Feytaud 1924; Vieau 2001) and recently demonstrated by molecular phylogenetic studies (Miura et al. 1998; Clément et al. 2001; Jenkins et al. 2001; Austin et al. 2002, 2004; Marini and Mantovani 2002; Ohkuma et al. 2004; Uva et al. 2004; Ye et al. 2004).

Material and methods

The termite *Reticulitermes grassei* from the southwest of France (Clément et al. 2001; Uva et al. 2004), treated as *R. lucifugus grassei* by other authors (Jenkins et al. 2001; Marini and Mantovani 2002), was collected in the area of Les Eyzies; *R. santonensis* (Clément et al. 2001; Uva et al. 2004) was collected on the Ile d'Oléron and cultured at the laboratory of the Université de Bourgogne in Dijon. Both were identified by morphological criteria (Clément 1978). Specimens of *R. flavipes* were provided by Prof. König from the University of Mainz (Germany); they originate from the Bundesanstalt für Materialforschung und Materialprüfung (Berlin, Germany). The hindgut of each studied termite was opened with a pair of tweezers, and the fluid content was mixed in a drop of Ringer's solution. Protozoa were observed and photographed under either phase contrast or differential interference contrast, using a Leica DMR microscope equipped with a Q-Fish Light Station.

For immunofluorescence, cells were permeabilized in 0.5% Triton X-100 in Tris-maleate buffer for 1 min, and air-dried on immunofluorescence slides previously coated with 0.1% poly L-lysine solution (Sigma). Alternatively, the Triton-permeabilized cells were fixed with 3.7% formaldehyde for 5 min before drying on the poly L-lysine-coated immunofluorescence slides. Slides were stored at −20 °C. After two on-hour washes in PBS, the cells were blocked with 1% bovine serum albumin for 15 min, and incubated overnight at 4 °C with the undiluted supernatant of the monoclonal antibodies (MAb). After three on-hour washes in PBS, the cells were incubated with a 1/200 dilution of the secondary antibody, an anti-mouse Ig:IgG/M antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma). After washing twice in PBS for 1 h, the slides were mounted in 1/1 PBS/glycerine solution containing 10 mg/ml DABCO (Sigma) as an antifading agent. The MAb used were: the anti-tubulin MAb IG10, the anti-parabasal fiber MAb IE10, and several 3H5, 24E3, 2G5, and VIF1 MAbs produced against cytoskeletons of trichomonads such as *Trichomonas vaginalis* or *Tetratrichomonas gallinarum* by the first author according to the procedure described by Brugerolle and Viscogliosi (1994).

For transmission electron microscopy (TEM), the entire fauna of the termite gut was fixed in a solution of 1% glutaraldehyde (Polysciences) in 0.1 M phosphate buffer at pH