An immunofluorescence study of mitosis in a mite-pathogen, *Neozygites* sp. (*Zygomycetes: Entomophthorales*)

T. M. Butt and R. A. Humber*

USDA-ARS, Plant Protection Research Unit, US Plant, Soil, and Nutrition Laboratory, Ithaca, New York

Received February 23, 1989
Accepted April 3, 1989

Summary. Mitosis in a mite-pathogenic species of *Neozygites* (*Zygomycetes: Entomophthorales*) was investigated by indirect immunofluorescence microscopy using an antibody against α-tubulin for visualization of microtubules (MTs). DAPI and rhodamine-conjugated phalloidin were used to stain chromatin and actin, respectively. Salient features of mitosis in *Neozygites* sp. are (1) a strong tendency for mitotic synchrony in any given cell, (2) conical protrusions at the poles of metaphase and anaphase nuclei revealed by actin staining, (3) absence of astral and other cytoplasmic MTs, (4) a spindle that occupies most of the nuclear volume at metaphase, (5) a spindle that remains symmetrical throughout most of mitosis, (6) kinctochore MTs that shorten during anaphase A, (7) a central spindle that elongates during anaphase B, pushing the daughter nuclei into the cell apices, and (8) interpolar MTs that continue to elongate even after separation of the daughter nuclei. Cortical cytoplasmic MTs are present in a few interphasic and post-cytokinetic cells. The data presented show that *Neozygites* possesses features unique to this genus and support the erection of the *Neozygitaceae* as a separate family in the *Entomophthorales*.

Keywords: *Neozygites* sp.; Mitosis; *Entomophthorales*; Immunofluorescence; Microtubules.

Abbreviations: DAPI 4,6-diamidino-2-phenylindole; MT microtubule; SPB spindle pole body.

Introduction

The *Neozygitaceae* was recently separated from other entomophthoralean fungi (Ben-Ze'ev et al. 1987) on the basis of relatively few data on the cytology of mitotic and non-mitotic nuclei of *Neozygites fresenii* (e.g., Butt 1983, and cited in Murrin et al. 1984 a). Since these and other entomophthoralean pathogens are considered potential agents for the biological control of agricultural pests, it is vital that these fungi should be classified correctly. One benefit to be gained from this is that certain predictions could then be made on several aspects of their biology and, perhaps, lead to more effective use of these important arthropod pathogens in pest control.

This study focusses on mitosis in a mite-pathogen, *Neozygites* sp., because mitosis and spindle pole bodies are considered to be useful indicators of taxonomic and phylogenetic affinities (Kubai 1978; Heath 1980 b-c, 1981, 1986). It complements an earlier study of the distribution of actin in *Neozygites* sp. (Butt and Heath 1988) and establishes the spatial-temporal relationships of actin, tubulin and chromatin during mitosis and cytokinesis. We provide a detailed account of mitosis in this genus and present data supporting the erection of this new family.

Materials and methods

Fungal strain and culture

The fungus used in this study is an unidentified species of *Neozygites* that attacks *Tetranychus urticae* Koch (*Acarina: Tetranychidae*) and other tetranychid mites in the eastern United States. A culture of this fungus was isolated in October, 1981, from an infected adult of *T. urticae* supplied by D. Smitley from an in vivo culture of infected mites maintained at the North Carolina State University, Raleigh. An infected mite containing numerous hyphal bodies was dissected in a drop of Grace's Insect tissue culture medium (GIBCO, Grand Island, New York) supplemented by 5% fetal bovine serum (GIBCO). This culture was deposited in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY) as ARSEF 662, and is now routinely grown in mammalian tissue culture Medium 199 (with Hank's salts; GIBCO) supplemented by 26.68 g/l sucrose and 5% fetal bovine serum.
**Immunofluorescence microscopy**

Spindle microtubules (MTs) can be visualized by indirect immunofluorescence microscopy using a monoclonal antibody raised against yeast α-tubulin (Kilmartin et al. 1982). This approach was necessary in our study because all the electron microscopy protocols we tried failed to preserve spindle MTs.

Cells in mid-log phase were fixed by adding 1 ml of freshly prepared 30% (w/v) formaldehyde to a 9 ml culture. The culture was gently agitated during fixation for 40 min, then harvested in a Sorvall RC-5 B centrifuge (DuPont) using a SS-34 rotor (746 g for 6 min). After several washes with phosphate-buffered saline (PBS), pH 7.4, the cells were permeabilized with 1 mg/ml buffered-Novozym 234 (Novo BioLabs) for 10 min, rinsed in buffer, and exposed to 0.1% (v/v) PBS-Trition X-100 for 10 min. The cells were transferred to Eppendorf tubes and incubated 4 h or overnight at 37 °C with YOL 1/34 antibody against α-yeast tubulin (a gift of J. Kilmartin, Cambridge, U.K.) diluted 1:100 in PBS. Sodium azide was added at a final concentration of 0.01% when incubated overnight. Incubation in rhodamine-conjugated secondary antibody (rabbit anti-rat; Miles Laboratories), diluted 1:40 in PBS, was for 3–6 h at 37 °C. Cells were washed extensively before and after incubation with primary and secondary antibodies. Cells were mounted in a polyvinyl alcohol–glycerol mounting medium containing p-phenylenediamine (Johnson and Naoguera-Araujo 1981). All preparations were examined in an Olympus BH-2 microscope equipped with epifluorescence illumination, and standard UV, FITC and rhodamine filter sets. Images were recorded on Kodak Tri-X or TMAX 400 film.

**Visualization of actin and DNA**

Cells were fixed and stained for actin and DNA according to Butt and Heath (1988), except that the nuclei were stained with 10 μl of a 1 mg/ml aqueous solution of 4,6-diamidino-2-phenylindole (DAPI; Sigma) rather than mithramycin.

**Results**

**Interphase**

A description of the in vitro cells of Neozygites sp. is given in Butt and Heath (1988). Approximately 90% of the 500 cells examined in a log phase culture were in interphase. Nuclei in any one interphase cell were typically uniform in size, evenly spaced in the cytoplasm, and encased in an intensely staining shell of actin (Butt and Heath 1988).

The dispersed, uniform DAPI staining throughout the nucleoplasm suggests that the interphasic chromatin was diffuse rather than condensed into discrete aggregates (Fig. 1 a). Nucleoli appeared as non-fluorescing regions of the nucleus (Fig. 1 a). Most nuclei possessed a single, prominent, spherical (rarely lobed) nucleolus, 1.7 ± 0.4 μm in diameter, located centrally or peripherally (Fig. 1 a).

**Prophase**

Mitosis was often initiated at one end of the cell and proceeded to the other, ultimately becoming synchronous (Fig. 1) or nearly synchronous (Fig. 2). So few nuclei were observed in prophase that this stage was assumed to be fairly brief. Prophase nuclei were identified by the reticulate pattern of the condensing chromatin, and the formation of intranuclear MTs (Figs. 1 and 2). The latter did not emanate from any one

---

**Fig. 1 a, b.** Cells in interphase (I), prophase (P), and metaphase (M), with all the mitotic nuclei in synchrony. a DAPI; interphase nuclei contain non-fluorescing nucleoli. The chromatin in prophase nuclei is reticulate while that in metaphase nuclei is mostly aligned at the equator with only a few chromatid arms trailing. b MT-staining: the MTs in prophase nuclei are organized into bundles aligned along the presumptive spindle axis. The metaphase nucleus contains a typical mid-metaphase spindle with the MTs converging at the apices. × 1740

**Fig. 2 a, b.** Metaphase cell in asynchronous mitosis. a DAPI; the first nucleus (left hand side) is in prophase, the last is in metaphase. b MT-staining; the spindle during early metaphase is typically barrel-shaped. × 1840

**Fig. 3 a, b.** Asynchronous mitoses. a Rhodamine-conjugated phalloidin; perinuclear actin reveals the nuclear shape; note polar deformations of the nuclear envelope (arrow) on the anaphase nucleus. b DAPI staining of the same cell shown in a, the two upper nuclei are in metaphase, the two lower in early anaphase. × 1560

**Fig. 4 a, b.** Two focal planes of nuclei in synchronized early anaphase. All the MTs converge at the pole; the kinetochore MTs are interspersed amongst the interpolar MT bundles (arrow) and appear as a brightly fluorescing apical cone. Note that the ends of the spindle are pointed. × 1720

**Fig. 5 a–c.** Mid-anaphase. a Rhodamine-conjugated phalloidin; fortuitous staining of perinuclear actin defines the shape of anaphase nuclei. Note the polar protusion (arrow) of the nuclear envelope is similar to that visualized during metaphase. b The same cell but at a different focal plane. c DAPI staining of the same cells shown in a and b. Chromatin is absent from the attenuated central portion of the dividing nucleus seen in a. × 1550

**Fig. 6 a, b.** Mid-anaphase. a DAPI; chromatin masses are fully segregated and stain less intensely. b MT staining. Nonkinetochore MTs are highly conspicuous especially in the attenuated portion of the nucleus. × 1770

**Fig. 7 a–c.** Late anaphase nuclei. a, b Two focal planes showing the absence of prominent kinetochore MTs at the spindle poles, and possible overlapping of interpolar MTs in the central portion of the nuclei. c Corresponding DAPI staining. × 1800

**Fig. 8 a, b.** Telophase cell. a MT staining: helicoidal appearance of the interpolar MT bundles. b DAPI; the fusiform nuclei are concentrated in the cell apices. × 1660