ACTA PROTOZOOLOGICA

NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY
WARSAW, POLAND
1997
VOLUME 36 NUMBER 4
ISSN 0065-1583

Polish Academy of Sciences
Nencki Institute of Experimental Biology
and
Polish Society of Cell Biology

ACTA PROTOZOOLOGICA

International Journal on Protistology

ACTA PROTOZOOLOGICA appears quarterly.

The price (including Air Mail postage) of subscription to ACTA PROTOZOOLOGICA at 1998 is: US $ 180.- by institutions and US $ 120.- by individual subscribers. Limited number of back volumes at reduced rate are available. TERMS OF PAYMENT: Cheque, money oder or payment to be made to the Nencki Institute of Experimental Biology. Account Number: 11101053-3522-2700-1-34 at Państwowy Bank Kredytowy XIII Oddz. Warszawa, Poland. WITH NOTE: ACTA PROTOZOOLOGICA! For matters regarding ACTA PROTOZOOLOGICA, contact Managing Editor, Nencki Institute of Experimental Biology, ul. Pasteura 3, 02-093 Warszawa, Poland; Fax: 48-22 8225342; E-mail: jurek@ameba.nencki.gov.pl (for more information see web page **http://www.nencki.gov.pl/public.htm**).

Front cover: *Raphidiophrys pallida*. *In*: J.J.Lee, S.H.Hunter and E.C.Bovee, Editors - An illustrated guide to the protozoa Society of Protozoologists 1985

This publication is supported by the State Committee for Scientific Research
Printed at the MARBIS, ul. Kombatantów 60, 05-070 Sulejówek, Poland

Acta Protozoologica (1997) 36: 237 - 248

ACTA
PROTOZOOLOGICA

Random Movements of Soil Amebas

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Summary. Visual assays and videomicroscopy were used to record and analyze movements of soil amebas in the absence of chemical signal gradients. Seventeen isolates of amebas belonging to 8 genera, including several potential pathogens, were studied. Videorecordings of movements on glass or plastic surfaces in saline solutions or liquid media were made over periods of 15-60 min, and tracks of individual cells were then traced at intervals of 1 or 2 min onto acetate overlay sheets. Mean and variance of speed and turning angle were computed, as well as the McCutcheon index (total displacement from the starting point divided by the distance traveled) and the mean square displacement. The absolute value of the angle of change in direction during intervals of 1 or 2 min had in all cases a mean less than 60 degrees. Dispersion appeared to follow a power law: in most cases the logarithm of the mean square displacement plotted against the logarithm of time gave a straight line with slope between 1 and 2, usually closer to 2, over periods ranging from 15 to 60 min. The theory of the random walk predicts that in a Gaussian random walk the slope should be 1, whereas a slope of 2 would be expected in the case of "ballistic dispersion." Thus, in the time scale of these experiments, the amebas dispersed more rapidly than would be expected from a Gaussian model. This would be an advantage in populations searching for food. We suggest that the random movements of soil amebas may be approximated by a 1-parameter class of self-similar random processes termed Levy walks. These include both Gaussian and ballistic dispersion as special cases at opposite extremes of the parameter's range.

Key words: *Acanthamoeba*, amebas, ameboid motion, *Dictyostelium*, fractals, Levy walk, random walk.

INTRODUCTION

Small amebas are a significant component of the ecology of many soils, where they feed on bacteria and other microbes (Singh 1975, Pussard et al. 1994). Some species can also infect animals, including humans, causing serious disease (Martinez and Visvesvara 1997). Given their ecological and medical importance, an understanding of their motility and behavior is potentially valuable. Several studies have documented behavioral responses to potential chemical signals from bacteria or animal tissues (McIntyre and Jenkin 1969, Cline et al. 1986, Marciano-Cabral and Cline 1987, Schuster et al. 1993). In a recent study of *Acanthamoeba castellanii* chemosensory responses we observed that, in the absence of added chemical signals, this species disperses randomly in a manner somewhat different from that predicted by the classical Brownian motion or Gaussian diffusion model (Schuster and Levandowsky 1996). In the present paper we extend these observations to other species of amebas and examine the basic question of motility in a uniform

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medium (i.e., in the absence of gradients of chemical signals).

One reason for studying statistical details of random motion is that mathematical models have been proposed for the population behavior of small ameboid cells, such as cellular slime molds and leukocytes (Keller and Segel 1970, Zigmond 1977, Okubo 1980, Lauffenberger et al. 1983). In these it is assumed that, in the absence of a sensory signal, cells would disperse in a random walk. Mathematically, this assumption is usually modelled by a standard diffusion term, and the motion of an individual cell would be approximated by a random walk in which displacements follow a Gaussian, or normal distribution. In this kind of dispersion, the standard deviation of the spatial distribution of a population of cells increases as the square root of time (Berg 1993).

Such movement, though, may not be optimal for a cell searching for food, as it would lead to repeated sampling of the same area when the cell crosses its back trail (Levandowsky et al. 1988a,b). Thus, a more plausible model might be based on a random walk with a different type of statistics, where such repeated sampling would occur less often than with Gaussian diffusion.

MATERIALS AND METHODS

The following organisms were used: *Naegleria gruberi* 1518/1, EG_B (Berkeley isolate), BZ21 (Brazilian isolate), CR54 (Costa Rican isolate), *Acanthamoeba astronyxis*, *A. culbertsoni* (A-1, ATCC 30171), *A. castellanii* (Neff strain, and VO14), *A. palestinensis*, *A. polyphaga* (Texas isolate ATCC 30461, and VO29), *A. rhysodes*, *Leptomyxa reticulata* (Pussard strain), *Tetramitus rostratus* (ATCC 30216), *Balamuthia mandrillaris (V194)*, *Phreatamoeba balamuthi* (ATCC 30984), *Dictyostelium discoideum* (ATCC 11735), and unidentified vahlkampfid Indonesian isolate (IND-3). Several of these are potential pathogens, including *A. culbertsoni* (pathogenic for mice), *A. polyphaga*, (isolated from corneal infections), and *B. mandrillaris*, (human isolate from a case of amebic encephalitis).

Acanthamoeba spp. were grown axenically in PPYG medium (proteose-peptone 2% w/v, yeast extract 0.5%, glucose 0.5%) or in PPYG supplemented with 5% newborn calf serum and 1% MEM vitamins solution (Sigma). *Acanthamoeba castellanii* (Neff) and *T. rostratus* were also grown on bacterized PYG medium (proteose peptone 0.05%, yeast extract 0.05%, glucose 0.1%, agar 1.5%) with *Klebsiella pneumoniae* as a food source. *Naegleria* was grown axenically in a medium containing proteose-peptone 0.25%; yeast extract 0.25%; liver concentrate 0.5%; and calf serum 7.5%. *B. mandrillaris* was cultivated axenically in a complex growth medium (Schuster and Visvesvara 1996). *Phreatamoeba balamuthi*, an anaerobic ameboflagellate, was grown in fluid thioglycollate medium (Difco). *Leptomyxa reticulata* was grown in WB saline (Table 1) with *Escherichia coli* as a bacterial food source. *D. discoideum* was grown in half-strength fluid peptone-yeast medium with *E. coli* as a food source.

Table 1. Components of saline solutions (grams/liter)

	Page Saline	WB Saline	Hanks' Balanced Salts
NaCl	0.12	0.58	8.00
$MgSO_4 \cdot 7H_2O$	0.004	0.49	0.10
$MgCl_2 \cdot 6H_2O$	-	-	0.10
$CaCl_2$	0.004	-	0.14
$CaCl_2 \cdot 2H_2O$	-	0.04	-
KCl	-	-	0.40
KH_2PO_4	0.14	0.54	0.06
Na_2HPO_4	0.14	0.14	0.48
$NaHCO_3$	-	-	0.35
D-glucose	-	-	1.00
$Na_2EDTA \cdot 2H_2O$	-	0.13	-
pH	7.0	6.5	7.0

For axenic cultures, logarithmic-phase cultures grown in Corning tissue culture flasks (25 cm^3) at 25°C, were harvested by chilling the flasks on ice, followed by several sharp taps against the lab bench to dislodge the amebas from the flask surface. For bacterized populations on agar, the surface of a 24-36 h culture was flooded with cold saline solution and the amebas removed from the agar with a sterile glass spreader rod. Amebas were then washed with cold saline to remove bacteria. Table 1 gives compositions of the saline solutions used both for washing and for experimental manipulation. Page (1967) and WB saline solutions were used for *Acanthamoeba* spp., *N. gruberi*, *T. rostratus*, *D. discoideum* and the Indonesian isolate IND-3. Hank's balanced salt solution was used for *B. mandrillaris* because of its greater osmotic sensitivity. *P. balamuthi*, because of its need for reducing conditions, was observed, without washing, in tissue culture flasks filled with thioglycollate medium to exclude air. *Leptomyxa* isolates were examined in their tissue culture flasks after gentle washing of the flask with WB saline to remove residual bacteria.

Two assay methods were used for tracking random movements. In the first, amebas were collected by centrifugation and their numbers adjusted after counting in a Coulter Counter to give approximately 10^6 amebas/ml. Aliquots of 20 µl of the suspension (*ca* 20,000 amebas) in growth medium were placed on rectangular coverslips (24 x 30 mm) and allowed to settle for at least 20 min, during which time the trophic amebas attached to the substrate. Amebas from bacterized cultures, washed in saline, were suspended in peptone-yeast medium in order to facilitate attachment of amebas to the coverslip surface. Attachment in saline solution was poor. At the start of an experiment, culture fluid was drained using absorbent tissue. The coverslip was then gently but thoroughly washed by repeated dipping in a large volume of dilute saline solution to eliminate growth medium, drained again of excess fluid and mounted on a Plexiglass chemotactic slide chamber (Zigmond 1988) so that the amebas were suspended over the bridge connecting the two wells of the slide (Fig. 1). The coverslip was held in place at either end by metal clips. The width of the bridge under the amebas was 1 mm, and the space between the coverslip and the bridge was estimated to hold 1 µl of fluid. Each of the two chambers received an aliquot of 130 µl of saline solution. In several experiments with *A. castellanii*, the same concentration of an attractant, lipid A (0.01 mg/ml) (Schuster and Levandowsky 1996) was introduced into both chambers. The slide was

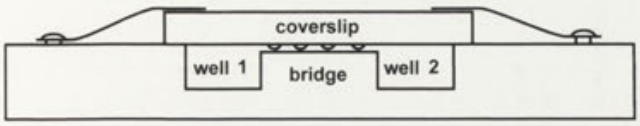

Fig. 1. Schematic representation of visual assay slide (Zigmond chamber). Amebas are attached to the inverted coverslip mounted over the bridge (the space between the coverslip and bridge is exaggerated for clarity). Saline solution or other fluid is placed in the two wells and fills the space under the coverslip

placed on the stage of an inverted microscope at room temperature (*ca* 25°C; a heat filter was used in the microscope, and heating from the light appeared to be negligible) and viewed through a 10x or 20x phase-contrast objective.

In the second method, used in some experiments with *A. castellanii, Leptomyxa reticulata* and *Phreatamoeba balamuthi*, cells were observed directly and recorded as they moved on the wall of the tissue flask in their culture medium, or after the culture medium had been replaced with saline solution. Cell size was a factor in determining the assay method. Larger amebas, like *Leptomyxa*, could be studied only in tissue culture flasks. *Phreatamoeba balamuthi*, which requires anaerobic conditions, was studied in fluid thioglycollate medium.

Movements of amebas were followed on a videomonitor for periods ranging from 10 to 35 min and recorded on videotape using a time-lapse videorecorder. Using the recording, cell outlines were traced at successive 1 or 2 min intervals from the videomonitor (videoscreen magnification of 270x) using a clear acetate sheet and a felt-tipped pen. The moving ameba was followed until it moved off the screen, or the taped sequence came to an end. Elapsed time was determined from the readout on the videoscreen and averaged about 20 min, with a range of 15-60 min. For each experimental run 5 or more amebas were randomly chosen from a field of about 50. The criteria for inclusion were that the ameba moved (on the average, about 10% of amebas were nonmotile), did not collide with other amebas in the field and did not undergo cytokinesis during the time of observation.

Centroids of successive cell outline tracings were judged by eye and these were connected with straight lines to obtain an approximation of the cell's path. From this track, the total path length of the ameba was determined by summing the length of the connecting straight lines. Knowing the elapsed time, the average speed of the individual cell was calculated. In addition, the displacement (straight line distance from starting point to end point) was measured at successive times, and used to calculate the mean square displacement of a number of cells with time. The logarithm of the mean square displacement was plotted against the logarithm of time, and the slope of the resulting straight line was measured. This is the Levy slope. The McCutcheon index was also calculated (McCutcheon 1946). This is the ratio of the displacement (straight line distance from starting to end point) to the total path length (Fig. 2). This ratio ranges from 0 (the cell returns to its starting point) to 1 (the cell goes in a straight line).

Turning angles were obtained by connecting the centroids of successive cell tracings with straight lines and measuring the angles between successive straight lines.

RESULTS

Representative tracks of amebas are shown in Figs. 3a,b. In most cases amebas moved with minimal turning, rarely crossing their earlier paths. This can also be seen in

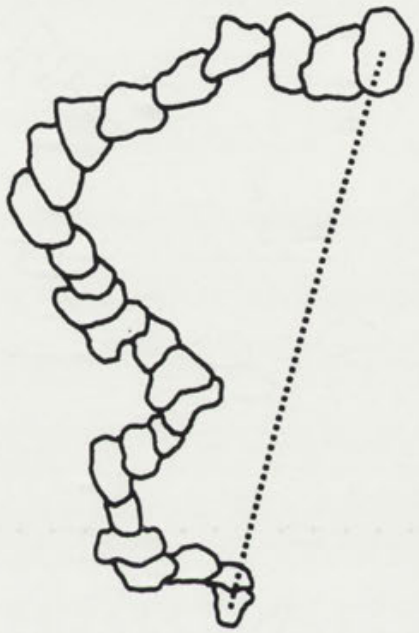

Fig. 2. The McCutcheon ratio or index is given by the net displacement (length of dotted line) divided by the length of the actual path (shown by tracings of cell outlines at intervals of 1 min)

the statistics of the absolute values of the turning angles, where the means were relatively low, in many cases not significantly different from zero (Table 2), and angles greater than 90 degrees were relatively uncommon (Fig. 4).

The Levy slope was greater than 1 for all strains tested, indicating a faster than Gaussian dispersion rate (Table 2). In several cases it approached the theoretical maximum of 2 ("ballistic" dispersion). It varied for the same species in different experiments. Thus, for *A. castellanii* it ranged from 1.5 to 1.9. All the species tested fell in this range, and none was close to the theoretical value 1, corresponding to a true Gaussian diffusion. Fig. 5 shows representative plots.

Tetramitus amebas and some strains of *Naegleria gruberi* were among the fastest of the amebas tested, with speeds of 1.1-1.8 μm/s. Some *Naegleria* isolates however were much slower, 0.16-0.34 μm/s (Table 2). *Naegleria* amebas have a monopodal or limacine type of movement, very different from the movement of *Acanthamoeba*, but

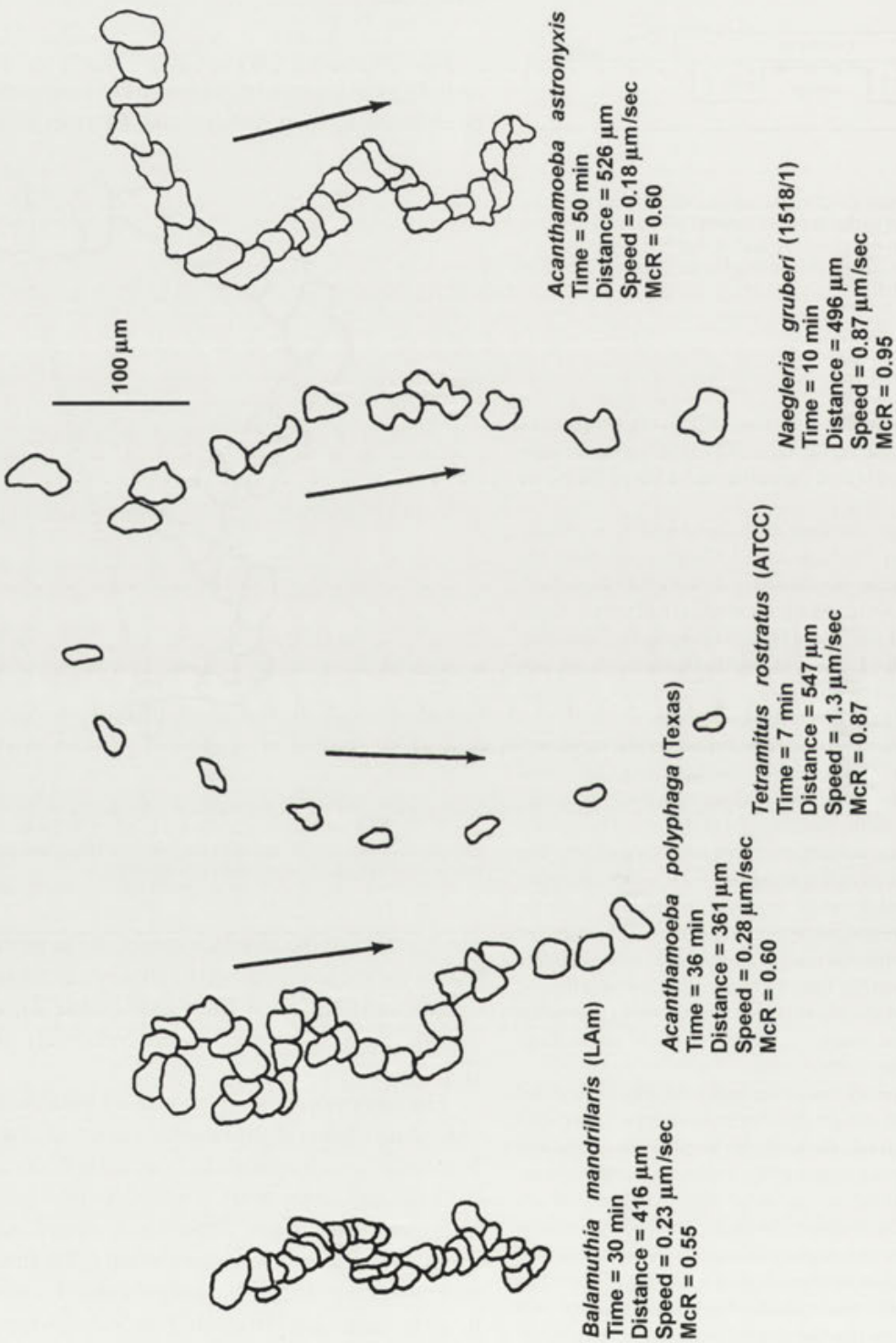

Fig. 3a. Tracings of cell outlines at 1 min intervals, showing representative tracks of isolates with comparable size but varying speeds

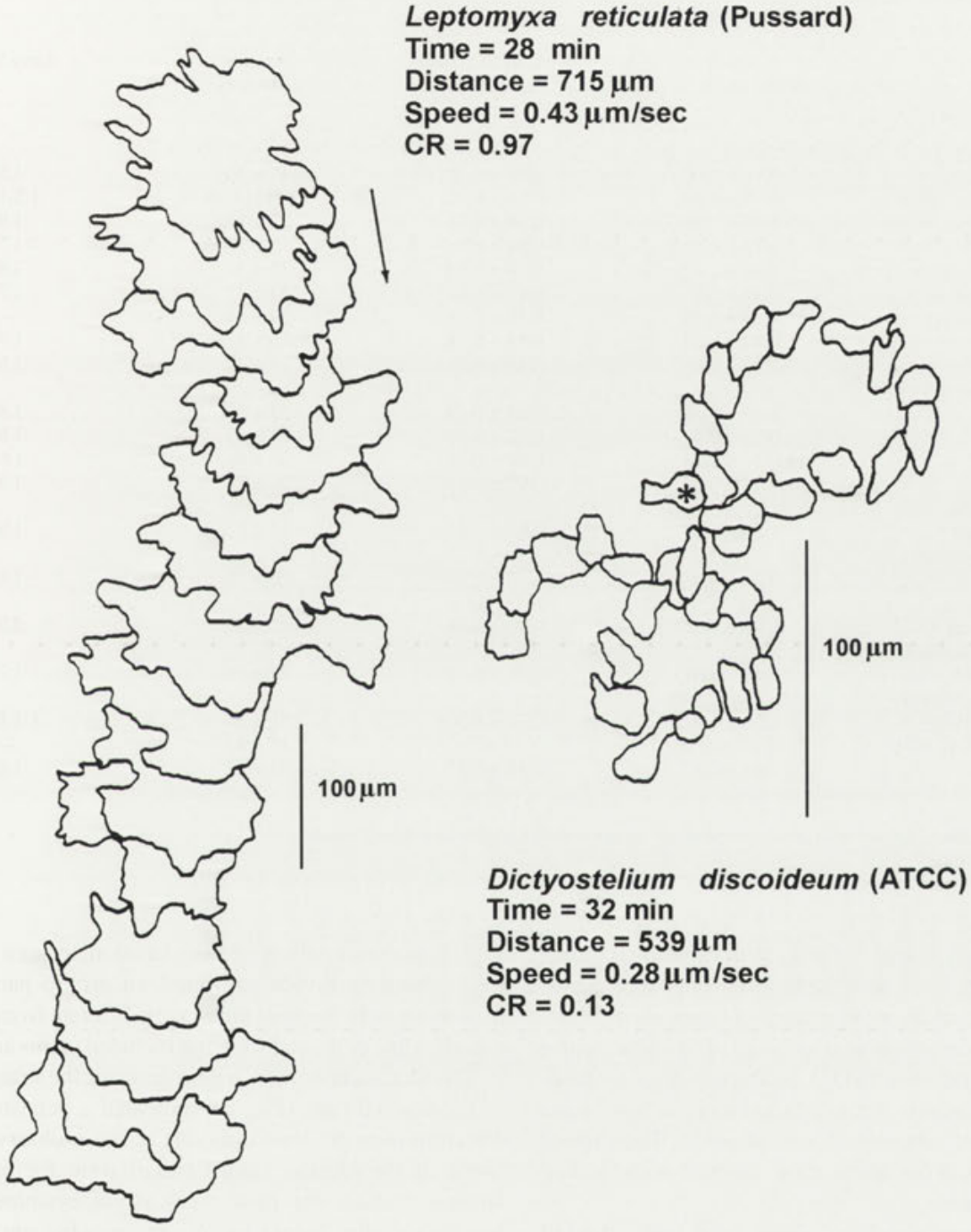

Fig. 3b. Comparison of amebas with very different sizes, which also contrast greatly in McCutcheon ratio and Levy slope. Asterisk shows the starting point

limacine movement in itself was not a cause of faster movement, since some *Naegleria* moved comparatively slowly. We include also the movement of *A. castellanii* (Neff) in both saline and lipid A solutions (Table 2). Lipid A is a chemoattractant (Schuster and Levandowsky 1996) but was presented here in a uniform concentration, without spatial gradients. Speed was identical in the both saline and lipid A solutions, and the McCutcheon ratio and Levy parameter were also similar.

The speed of movement of *Acanthamoeba* spp. was generally within a narrow range (0.25-0.45 µm/s). *A. astronyxis*, a larger species, moved at a slower rate.

Table 2. Parameters of Motion

	Speed (μm/second ± S.D.)	McCutcheon Index (± S. D.)	Angular + Changes	Levy Slope
Acanthamoeba spp.				
A. astronyxis	0.16 ± 0.07	0.48 ± 0.21	45 ± 30	1.5
A. castellanii (Neff)	0.39 ± 0.09	0.64 ± 0.22	19-51±18-38	1.5-1.9
- lipid A/lipid A	0.39 ± 0.09	0.58 ± 0.21	32 ± 22	1.9
A. castellanii (V014) **	0.25 ± 0.03	0.64 ± 0.16	15 ± 23	1.7
A. culbertsoni (A-1) **	0.42 ± 0.12	0.70 ± 0.14	37 ± 30	1.8
A. palestinensis	0.28 ± 0.03	0.60 ± 0.23	33 ± 35	1.7
A. polyphaga (Texas) **	0.34 ± 0.09	0.29 ± 0.16	29 ± 44	-
A. polyphaga (V029) **	0.42 ± 0.08	0.68 ± 0.21	13 ± 17	1.9
A. rhysodes	0.35 ± 0.07	0.49 ± 0.21	17 ± 32	1.6
Naegleria gruberi				
BZ 21 *	1.30 ± 0.29	0.83 ± 0.09	33 ± 55	1.8
EG_B	0.34 ± 0.04	0.52 ± 0.12	31 ± 35	1.8
CR 54	0.16 ± 0.04	0.48 ± 0.18	39 ± 42	1.6
1518/1	1.10 ± 0.14	0.97 ± 0.02	26 ± 21	1.9
Tetramitus rostratus				
ATCC 30216 *	1.80 ± 0.37	0.94 ± 0.03	17 ± 17	1.9
Balamuthia mandrillaris				
LAm (V194) **	0.24 ± 0.03	0.62 ± 0.27	20 ± 21	1.8
Leptomyxa reticulata				
Pussard strain *	0.41 ± 0.05	0.93 ± 0.08	15 ± 27	2.0
Phreatamoeba balamuthi				
ATCC 30984	0.16 ± 0.01	0.79 ± 0.13	23 ± 26	1.7
Dictyostelium discoideum				
ATCC 11735 *	0.28 ± 0.07	0.38 ± 0.20	51 ± 44	1.4-1.6
Soll Data (Soll, 1988)	0.13 ± 0.07	—	35 ± 47	—
IND-3 Isolate *	0.64 ± 0.12	0.49 ± 0.13	53 ± 43	1.6

+ Mean and standard deviation of absolute values of successive turn angles in a typical track
* Bacterized culture
** Pathogen or potential pathogen

Greater variation in speed of *Naegleria* strains was found. *Tetramitus* (Fig. 3a), one of the fastest in our experiments, also produced rapidly moving feeding fronts when grown on a bacterial carpet on agar surfaces. The vahlkampfid isolate from Indonesia, IND-3, produced very slow feeding fronts on bacteria, yet individual amebas moved at a speed typical of other isolates (0.64 μm/s). Thus, speed of movement did not necessarily correlate with feeding activity in culture.

Dictyostelium amebas, about half the size of *Acanthamoeba* and *Naegleria* cells, exhibited more variability in measured parameters, with some populations showing incipient stages of aggregation to form pseudoplasmodia. *Dictyostelium* cells were more likely to cross their paths than the other soil amebas (e.g., Fig. 3b). This is reflected in the McCutcheon index of 0.38, the lowest of the amebas tested in this study (Table 2).

Because cells were from logarithmic phase cultures, cytokinesis was sometimes observed during experiments. With *Leptomyxa*, cells were also occasionally seen to fuse. Cells about to divide exhibited an erratic pattern of movement with frequent turns, very different from that of non-dividing cells, and were not included in this analysis.

The McCutcheon ratio, a ratio denoting the straightness of the amebal path (Fig. 2), indicated a persistence in direction over the time intervals of these observations. Some of the highest values (>0.9) were for limacine amebas, though the *Leptomyxa* strain examined also had high values. Values for *Acanthamoeba* spp. varied from 0.29 (a corneal isolate of *A. polyphaga*) to 0.70 (*A. culbertsoni*).

Anomalous results were obtained with *A. polyphaga* (Texas strain) and with one preparation of *D. discoideum*. In these, a plot of log mean square displacement versus log time did not yield a straight line (data not shown).

There appeared to be no qualitative difference in cell movements of *A. castellani* in the Zigmond chamber (first assay method) and in their culture flasks (second assay

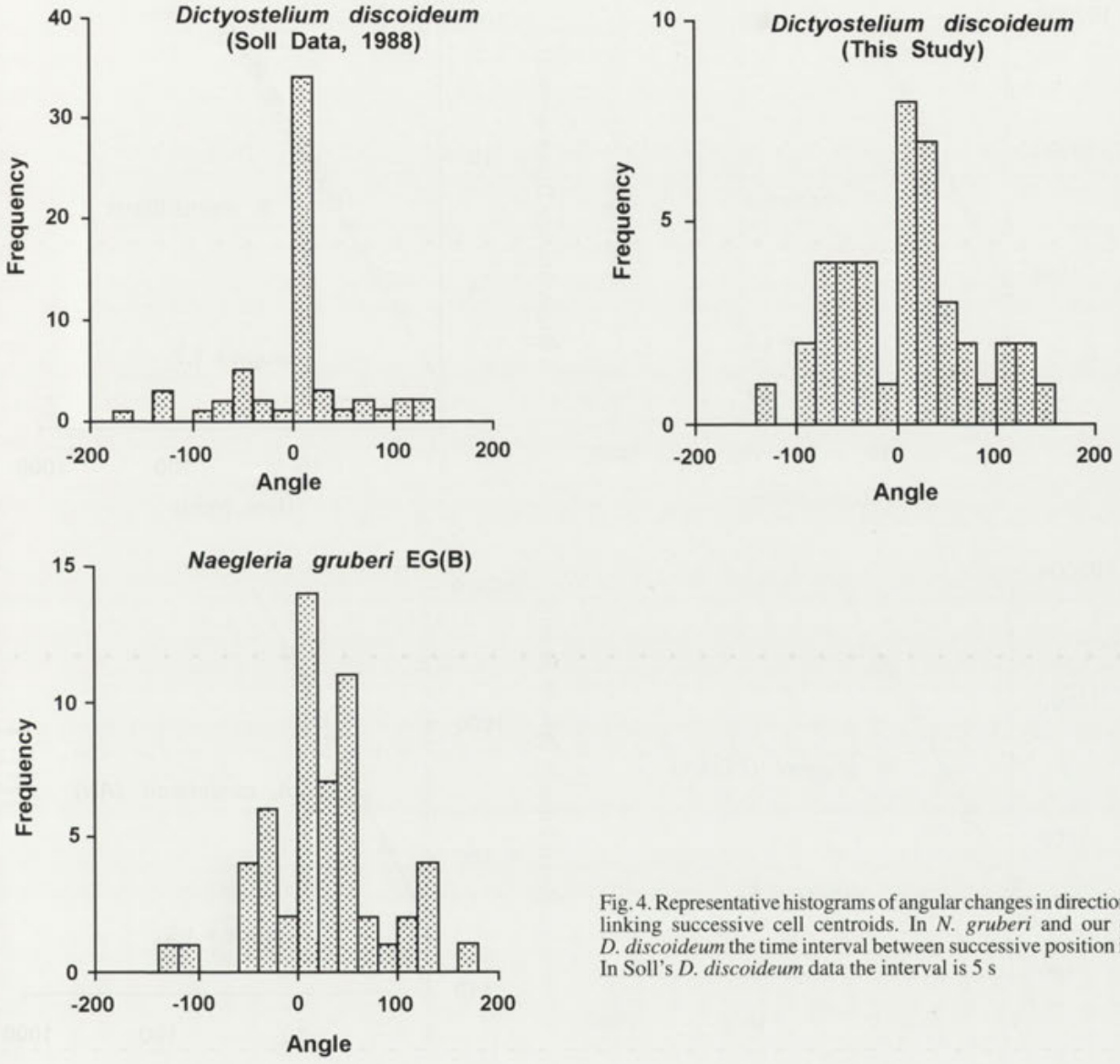

Fig. 4. Representative histograms of angular changes in direction in lines linking successive cell centroids. In *N. gruberi* and our data for *D. discoideum* the time interval between successive position is 1 min. In Soll's *D. discoideum* data the interval is 5 s

method). For cells moving in their growth medium, the results were similar to those obtained with the Zigmond chamber. For about an 1 h after the medium was replaced with Page saline (Page 1967), however, there appeared to be a period of physiological adaptation to hypo-osmotic saline solution. During this period, cells turned more often and the angles of the turns were more uniformly distributed over the interval (-180^0 - 180^0); the Levy slope was closer to 1 and the McCutcheon ratio was closer to 0 (data not shown). After about 2 h the cells turned less often and the Levy slope was closer to 2 and the McCutcheon ratio closer to 1. If WB saline (with higher osmolarity) instead of Page saline was used to replace the culture medium there was no period of changed motility.

DISCUSSION

The amebas used in these experiments constitute a variety of organisms of differing morphological, physiological and ecological types. They are all essentially bacterivorous organisms from soil and water habitats, though some are also opportunistic pathogens (Martinez and Visvesvara 1997). As such, the presence of a sensory

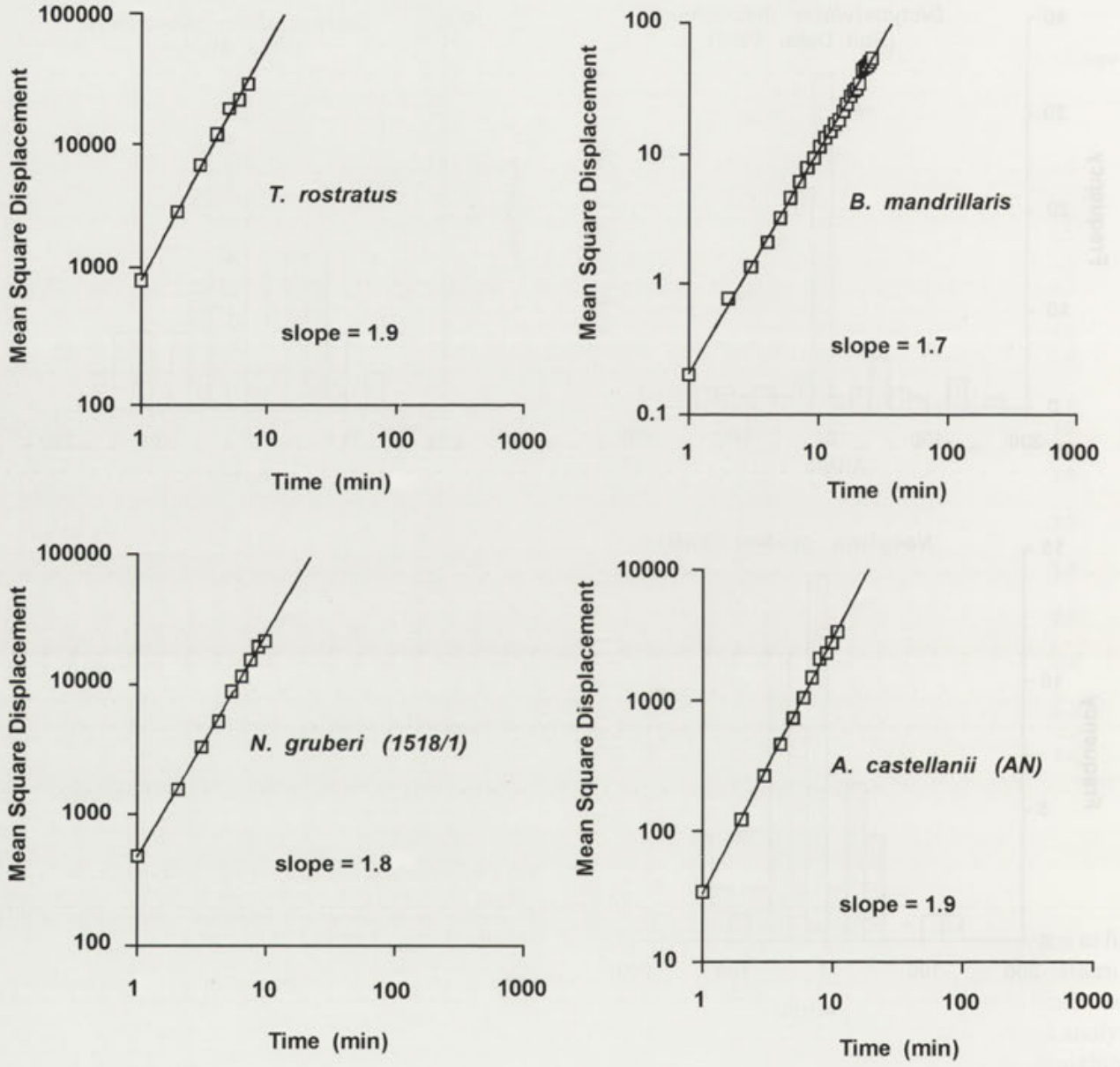

Fig. 5. Levy plots of representative amebas: logarithm of mean square displacement of 5 or more cells plotted against logarithm of time, and fitted with a straight line by least squares. Gaussian dispersion would give a slope of 1.0, and ballistic dispersion would give a slope of 2.0. Vertical scale units: for *N. gruberi, A. castellanii* and *T. rostratus* multiply by factor of 1.37 x 10 to obtain mean square displacement in μm^2; for *B. mandrillaris* the factor is 1.37×10^3

mechanism coupled with a search strategy for detecting bacteria would be advantageous. In this study we have focused on the basic search strategy in the absence of a gradient of potential chemoattractants: in saline solutions, in growth media, or in spatially uniform solutions of an attractant (lipid A).

Acanthamoeba spp. are a ubiquitous component of the soil microbiota and probably play an important role in regulating bacterial populations (Pussard et al. 1994). They are characterized by acanthopodia - short, finger-like pseudopods - that project from the cell surface, and show no obvious polarity in their locomotion. Several of the *Acanthamoeba* isolates that were examined in this study were pathogens or potential pathogens of humans. The role of these amebas in corneal infections, for example, may be augmented by the presence of bacteria

or by peptides released upon damage to the corneal surface (John 1991, Pfister et al. 1995). *Naegleria, Tetramitus* and *Phreatamoeba*, all genera of ameboflagellates, show a pronounced monopodal or limacine movement with an anterior eruptive pseudopod and a sticky uropod at the posterior end of the cell. *Phreatamoeba* requires anaerobic conditions for growth. *Balamuthia* and *Leptomyxa*, two additional genera that we have examined, are larger in size than members of the other genera. *Balamuthia* is known only as a pathogen from compromised hosts but probably occurs free in the soil environment. *Leptomyxa* may or may not be related to *Balamuthia*; the genera share some characteristics (similar type of cyst, nuclear morphology), but also have differences (Visvesvara et al. 1993).

In an earlier study we found that chemosensory responses of *A. castellanii* involved an increase in the McCutcheon, or chemotactic index, but not in the average speed (Schuster and Levandowsky 1996). This contrasts sharply with leukocyte behavior, in which both direction and speed change in response to chemical signals (Zigmond 1974, 1977). On the other hand Cline et al. (1986) found that tissue extracts did affect speed of movement of the ameboflagellate *Naegleria fowleri* in Boyden chambers (i.e., movement through a nitrocellulose filter). Unlike leukocytes, which do not move in the absence of stimuli (Wilkinson 1990), amebas are inherently active.

In the present paper we show that, even in the absence of a chemical signal, soil amebas tend to persist in their direction of motion, and do not make sudden turns and changes very often. This finding has implications regarding the speed with which a population of cells will disperse in the absence of chemical signals.

Cell dispersion models: Levy walks

The conventional model for random cell movements is Gaussian dispersion. This is the mathematical idealization of Brownian motion, whose probability distribution is the solution of the standard diffusion equation: that is, a Gaussian distribution (e.g., Berg 1993). This statistical model refers to the long-term, macroscopic (technically, asymptotic) behavior of a population, and it can occur as a result of many different microscopic kinds of detailed behavior (the terms macroscopic and microscopic are used here to refer to the long-term, asymptotic behavior on the one hand, and the detailed behavior on the scale of the observations on the other). The usual textbook example of a microscopic model leading to Gaussian dispersion is a series of straight line movements punctuated by random turns. In the simplest model the distances between turns (or, in the case of constant speed, the time intervals between successive turns) have an exponential distribution. This derives from the assumption that there is no "memory": the probability of a turn occurring at a given point (time) doesn't depend on the distance (time) since the last one. In a population of organisms undergoing such a random walk, the variance of the lengths of the straight line movements is finite, and the variance of the displacements of individuals from their original positions (the mean square displacement of the population) is proportional to the time elapsed. For long times the displacement has an approximately Gaussian distribution. This arises from the Central Limit Theorem of probability theory (Feller 1966).

The random walk just described is only one of many microscopic models that can give rise to Gaussian dispersion. They have the property that, over long enough times, the mean square displacement is proportional to time (e.g., Okubo 1980, Berg 1993). Gaussian dispersion, in turn, is one of a more general family of dispersion types (Levy 1937), in which the mean square displacement is proportional to time to an exponent between 1 and 2. These dispersion types can be achieved by random walk microscopic models, or Levy walks, like the one described above, with the difference that the lengths of straight line movements do not have finite variance.

Thus, Gaussian dispersion is at one extreme of a family of Levy dispersion processes. This is a 1-parameter family, in which the spatial variance increases in proportion to time raised to an exponent between 1 and 2 (a power law). At the other extreme is "ballistic dispersion," in which the mean square displacement is proportional to time with the exponent 2 (the variance of the displacement is proportional to time squared).

We refer here to the above microscopic models, in which straight line movements are punctuated by random turns and have a Levy distribution (a "stable distribution"), as Levy walks. The "memory-less" quality of the microscopic random walk model with exponential distribution of lengths (above) is absent from the Levy walks: in them, the probability of change in direction in the microscopic random walk model is influenced by the distance (time) since the last change.

In practice, one can distinguish among Gaussian, ballistic and Levy walks by plotting the logarithm of the mean square displacement of a population against the logarithm of time, and measuring the slope of resulting straight line plot. In the present case of soil amebas, for most of the isolates studied here such plots yielded a straight line with a slope closer to 2 than to 1, suggesting

that a Levy walk different from Gaussian dispersion may be a more useful model for the random motion of soil amebas. It has been suggested that, in organisms searching for food, Gaussian dispersion is less efficient than other Levy walks (Levy 1937; Schlesinger and Klafter 1985; Levandowsky et al. 1988a,b; Klafter et al. 1990). This is because the Gaussian random walk results in frequent resampling of areas already traversed.

Models leading to Gaussian dispersion

A number of workers have studied the movements of leukocytes or tissue cells in culture, and have fitted these data to models in which, for short times, the slope of log mean square displacement plotted against log time is close to 2, but over longer periods it is approximately 1. This difference in behavior in the short time scale is referred to as a time delay, or persistence. Thus, Gail and Boone (1970) found a persistence in direction in the random diffusion of mouse fibroblasts on agar surfaces. Over time periods of many hours, the effect of this persistence disappeared, and over a long enough period the dispersion was essentially Gaussian. The fibroblasts in their experiments, however, moved much more slowly than soil amebas, and we could not conduct our experiments for such long times, since the cells would leave the microscope field after a period of 15 min to 1 h.

Gruler and colleagues studied the statistical properties of motility in leukocyte preparations (Gruler and Bultman 1984, Gruler and de Boisfleury-Chevance 1987, Gruler 1993). They found that these relatively fast-moving ameboid cells follow a Gaussian dispersion with a short time delay, and calculated a diffusion constant. The pattern was clear after a period of only a few minutes in this case. A number of other cell dispersion models have been developed and analyzed which are essentially Gaussian over long time periods, but have a time delay, or persistence for short time periods (Alt 1990, Tranquillo 1990, Dickinson and Tranquillo 1993).

We do not know if the movement of soil amebas would appear Gaussian over a longer time period. As noted above, after a period between 15 min and an 1 h, all of our moving cells would have left the microscope field and thus we could not follow them for a longer time. During this time period, however, they moved many body lengths (Figs. 3a,b) and followed a pattern closer to ballistic than to Gaussian dispersion. In this time period they appear to fit the power law model of a Levy walk very well (Fig. 5).

From a strictly mathematical viewpoint, a true Levy walk has the fractal property of self-similarity, and one cannot tell from our data if soil ameba motion fits this requirement. Soll (1988) used an automated computerized system to track *Dictyostelium* cells, and published the track of a moving cell shown at 5 s intervals, with computer-generated centroids. In this one can see changes of direction that would be missed in our coarser analysis, which has a resolution of 1 min. We have measured the turning angles and McCutcheon index of 5 min of this published track. Comparison with our data for *D. discoideum* (Fig. 4, Table 2) shows some differences in these 2 time scales but the cultural conditions in Soll's study (axenic) were somewhat different from ours (bacterized), as were the average speeds (Table 2). Clearly more data are needed to test the hypothesis of self-similarity.

Regarding spatial scale, the relevance of laboratory experiments to the natural setting of the organisms should also be considered. For practical reasons, we observed cells moving on flat 2-dimensional surfaces, and this may not be the best model for movement in a 3-dimensional soil or tissue environment. Thus, very long term experiments might be hard to interpret ecologically, or clinically.

Of interest were some exceptions to the general pattern. The cases where cells turned frequently at random angles, and dispersed very slowly as in the Gaussian model were: (1) a cell that was about to divide and was thus probably undergoing mitosis and cytokinesis, and (2) cells in flasks where nutrient medium had recently been replaced by Page saline, a solution of lower tonicity. These were probably adapting physiologically to the new conditions. Other exceptions were: the Texas isolate of *A. polyphaga*, and one *Dictyostelium* preparation. In these cases the plots of log mean square displacement versus log time did not approximate a straight line. Possibly the *Dictyostelium* preparation was changing to the aggregation-type behavior typical of this species, during the experiment. The reason for the anomalous behavior of the *A. polyphaga* strain is not known.

As noted above, it has been suggested that Levy walks with a slope nearer to 2 than to 1 would have adaptive value for organisms searching for food, in that they would avoid resampling areas already sampled, and this would be more efficient. From this point of view, it is interesting and surprising that the motility of *A. castellanii* in flasks containing growth medium is similar to that in saline, even though the former cells are presumably well fed and do not need to search for food. One might have predicted that they would move less, or more slowly, but this was not the case.

Different types of dispersion can have implications regarding the validity of models of underlying physiologi-

cal processes of ameboid movement (e.g., Lauffenberger and Horwitz 1996), in the sense that such models yield predictions regarding dispersion. With *A. castellanii* (our best studied case), we found that the slope varied from one experiment to another, in the range 1.5-2.0, again suggesting that different nutritional or other physiological states in experimental populations can influence the frequency of turning. Dispersive patterns have been studied in other free-living organisms. Kawakubo and Tsuchiya (1981) found that the spreading of a population of a swimming ciliate, *Paramecium*, followed a Gaussian power law over periods of up to 12 s. Viswanathan et al. (1996) concluded that the foraging flights of albatrosses may be Levy walks, on the basis of the power law distribution of flight times and resting times. Similar conclusions were reached for the fruit fly, *Drosophila*, again on the basis of the distribution of episodes of inactivity and flight behavior (Cole 1995). In neither of the latter two cases were actual flight paths observed however.

In summary, in the absence of known chemical signal gradients, a number of soil ameba isolates showed a tendency for cells to persist in their direction of random movement, and this resulted in a more rapid dispersion than that predicted by the Gaussian dispersion model. This may be related to the theory of the Levy walk, in which the ratio of the logarithm of mean square displacement to logarithm of time is a measure of whether dispersion is Gaussian, ballistic, or something in between these two extremes.

Acknowledgements. We thank Dr. G. S. Visvesvara for providing cultures of some isolates for this study, and Drs Wolfgang Alt, J. Klafter and Roger Prince for useful discussions.

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Received on 9th April, 1997; accepted on 21st July, 1997

Acta Protozoologica (1997) 36: 249 - 260

ACTA
PROTOZOOLOGICA

Protein Kinase Inhibitors Abolish Adaptative Cell Behaviour in *Tetrahymena*

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Summary. Studies on the chemosensory behaviour of the ciliated protozoan *Tetrahymena thermophila* showed that protein kinase inhibitors abolish the adaptative swimming behaviour of the cells by making them unable to respond to a chemoattractant. Using proteose peptone or insulin as chemoattractants, dose/response-curves could be constructed for a number of tyrosine kinase inhibitors leading to complete inhibition of chemoattraction for genistein as well as for tyrphostin A47 (both at 80 μM) whereas the negative control substances daidzein and tyrphostin A63, used at the same concentrations, had no effects when the chemosensory behaviour was measured by a two-phase assay for chemoattraction. The more general protein kinase inhibitor staurosporine used at 30 nM was also inhibiting adaptative cell behaviour. Measurements of swimming speeds of individual cells by video-microscopy showed, in general, a stimulatory effect on swimming rates at the same concentrations of inhibitors when an inhibition was observed of the adaptation process. The results suggest that phosporylation of amino acid residues in proteins is a crucial step in adaptative swimming behaviour leading to chemoattraction. The abolishment of adaptation by inhibitors of protein kinases is possibly due to an inhibitory effect on ciliary reversals as more persistent forward swimming was observed in the presence of these inhibitors.

Key words: adaptation, chemoattraction, protein kinase inhibitors, *Tetrahymena*.

Abbreviations: PP - proteose peptone, PTK - protein tyrosine kinase.

INTRODUCTION

Rapidly swimming cells, like the ciliated protozoa, offer an interesting opportunity to study the chemosensory behaviour involved in chemoattraction/repulsion in response to external chemical stimuli. The membrane ultrastructure of ciliates resembles that of the chemosensory neurones and the olfactory epithelium in mammals (Hufnagel 1992, Menco 1992). As in higher organisms, the cilia may have adapted a sensory role. Ciliates may therefore be viewed as "swimming receptors" where correlations between cellular behaviour, cell behaviour and molecular signal transduction events can be studied experimentally because many ciliates are easy to grow and handle in the laboratory (Leick et al. 1994, Wheatley et al. 1994).

Previous studies of the chemosensory behaviour of *Tetrahymena* have shown that this ciliate is chemoattracted to a range of different proteins, peptides, and amino acids (Almagor et al. 1981, Leick et al. 1996). In order to gain insight in the cellular (and molecular) events leading to

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chemoattraction metabolic inhibitors have been applied that might be putative specific blockers of enzymatic processes crucial for the cellular behavioural response. Previously, we observed that the protein synthesis inhibitor cycloheximide was an effective inhibitor of chemoattraction although the effect seemed to occur by an unknown mechanisms unrelated to its effect on protein synthesis (Leick and Hellung-Larsen 1985).

The present report describes the effect of a series of protein kinase inhibitors on cell behaviour when stimulated by chemoattractants. We have selected a series of protein tyrosine kinase inhibitors as well as the more general protein kinase inhibitor staurosporine because protein phosphorylation is a widely applied molecular mechanism involved in switch-like outputs in cells (Ferrell 1996) as well as in chemotaxis of smooth muscle cells (Shimokado et al. 1995). Studies of the impact of metabolic inhibitors on general cell motile behaviour (in the present case chemoattraction) is an important prerequisite to undertake biochemical studies of the target protein molecules involved and their role in the signal cascades implied. However, we have such studies in progress. Using the two-phase assay and the Zigmond chamber we have, in this study, studied the adaptative (temporal) element of the chemosensory response. Adaptation is a widespread feature of many chemotactic cells and is a temporal mechanism that represents a kind of short-term memory (Morimoto and Koshland 1991). Adaptation is here defined as a reversible elimination of the responsiveness of a cell caused by an adjustment of its sensitivity. This element reflects the ability of the cells to read and "remember" a certain concentration of a chemoattractant thereby repelling them from swimming towards lower concentrations (Leick et al. 1994) in a similar way as in chemotactic bacterial cells where adaptation controls the balance between counterclockwise and clockwise rotation of the flagellar motor (Morimoto and Koshland 1991). In bacteria clockwise rotation of the flagellar motor leads to a stop whereas counter-clockwise rotation means forward swimming. In ciliates these two processes are represented by normal ciliary beating (forward swimming) and by reversal of the ciliary beating that reverses the active stroke.

Our results show that, in the presence of most of the protein kinase inhibitors used, the cells show normal (or accelerated) forward swimming motility, however, they loose their ability to sense a chemical gradient of a chemoattractant leading to compulsive forward swimming.

MATERIALS AND METHODS

Materials

Genistein, staurosporine and bovine insulin were from Sigma Chemical Comp., Mo. USA. Tyrphostins were from Calbiochem-Novabiochem Cal., USA.

Growth of cells

Tetrahymena thermophila (strain B7) was grown at 21-28°C to early or mid-stationary phase in complex growth medium, PPYS: 0.75 % proteose peptone (Difco) containing 0.75 % yeast extract (Difco) and 1.5 % glucose, 0.001 M $MgSO_4$, 0.00005 M $CaCl_2$, and 0.0001 M ferric citrate, in thin-layer (1-2 cm) cultures without agitation in Fernbach flasks. For chemoattraction experiments, cells were collected by centrifugation at 500 x g for 2 min, washed twice and resuspended in 10 mM HEPES (N-[2-Hydroxyethyl] piperazine-N´-[2-ethanesulphonic acid]), pH 7.4 (adjustment of pH with KOH) to a cell concentration of 1.5-2 x 10^5 cells per ml for about 40 h (2 days). In some experiments the cells were then pre-treated as indicated with various concentrations of proteose peptone (PP) 0-8mg/ml for 15 min before they were analysed in behavioural assays.

Two-phase chemoattraction assay

The two-phase assay is a method to determine chemoattraction of a cell population of *Tetrahymena*. 1.5 ml of cells in suspension were layered on the top of 1ml of a lower phase of high density (5 % metrizamide) solution containing the chemoattractant. The cells respond to the gradient of the attractant established by diffusion in the interphase between the upper and lower phase. The control experiment contained no attractant in the lower phase.

The two-phase assay was carried out at 28°C as described by Koppelhus et al. (1994). The chemoattractant was dissolved in 1 ml 5% (w/v) metrizamide (2-[3- acetamido-5-N-methyl-acetamido2,4, 6-triiodobenzamido]-2-deoxyglucose) from Nycomed, Oslo, Norway, in 10mM HEPES, pH 7.2 in a disposable 1ml plastic cuvette . A 1.5 ml suspension of starved cells was carefully layered on the top of the 1ml metrizamide solution that was the lower phase in the cuvette. The increase in cell concentration in the lower phase was monitored automatically by following the optical density (OD_{600}) in the metrizamide phase (central beam in a 3 cm cuvette) in a thermostated spectrophotometer (Shimadzu UV-160). The geometry of the system was that the cells had to migrate 0.5 cm into the lower metrizamide phase before being monitored by the light beam. Generally, the cells migrated in a swarm leading to a uniform suspension of cells in the lower phase at the time of the maximal response which occurred within 30 min at 28°C. The explanation for the late OD decreases is that cells swim upwards (probably by negative geotaxis) immediately following chemoattraction.

Determination of swimming speed of individual cells

Swimming speed of individual cells in free solution was determined by video microscopy using a microscopic chamber set-up. The chamber set-up was a modified Zigmond chamber as described earlier using no solidifying substance (Leick et al. 1994). Swimming tracks on the video screen with a duration of two seconds were transferred onto transparencies from the screen of video-taped recordings of *Tetrahymena*

thermophila pre-treated as indicated in each experiment. The length (mm) of individual tracks were monitored using an electronic graphics calculator (Numonics Corp., USA) and the distribution of swimming speeds under various conditions were calculated.

RESULTS

Inhibition of adaptative cell behaviour by genistein, tyrphostins and staurosporine

Initially we observed that cells treated with the tyrosine kinase inhibitor genistein (80-100μM) blocked cell accumulation at a solidified chemoattractant made by dissolving the chemoattractant in 10% semisolid gelatine. The cells did not swarm around the solidified chemoattractant in the presence genistein in contrast to untreated controls. This observation suggested to us that it was most likely the temporal element of adaptation (chemokinesis) in the swimming behaviour that was lost in the presence of this compound. As seen in Fig. 1, direct visual observation of cells layered on the top of a solution in a test tube, containing a linearly increasing gradient of proteose peptone (PP) from top to bottom, migrate to the bottom of the tube as a swarm but refuse to do so in the presence of 100 μM genistein. In this test tube assay, the linear gradient was set up experimentally with a gradient mixer (see legend to Fig. 1) in contrast to the two-phase assay where the gradient was established spontaneously by diffusion. In order to investigate this observation in more detail, a series of quantitative measurements of chemoattraction of the cell population in a two-phase assay was carried out.

In Fig. 2A it is shown that cells can be kept (adapt) in one concentration of the chemoattractant PP and still migrate to a higher concentration of this attractant. For example, cells placed in a solution of 0.8 mg/ml PP will adapt to this concentration and be attracted by the 1mg/ml phase. It was observed in Fig. 2A that cells adapting to 0.2 mg/ml PP responded more efficiently in the two-phase assay than control cells swimming in buffer solution. As swimming rates increase dramatically when buffer-starved cells are transferred to 0.2mg/ml PP it also increases the efficiency of the chemosensory response towards higher concentrations. In contrast, when PP-adapted cells were responding to a HEPES-buffer solution (starvation medium) they were repelled as seen in Fig. 2B.

Figure 3A shows that genistein interferes with the cell's ability to adapt. Genistein-treated cells refuse to respond by chemoattraction towards PP in the two-phase assay in a similar way as in the unstimulated control. In contrast, cells treated with 110 μM daidzein, an inactive analogue of genistein, respond normally towards PP (Takuma et al. 1996).

The protein kinase inhibitor staurosporine (42 nM) also abolished chemoattraction towards PP (Fig.3B). Staurosporine is a potent inhibitor of cAMP-dependent protein kinases as well as protein tyrosine kinases at 5-10nM in HeLa S3 cells (Tamaoki 1991). Staurosporine, used at about 20nM, induces death within 30 h in *Tetrahymena thermophila* in defined growth medium (Christensen et al. 1997, Straarup et al. 1997). We observed that cells treated with 42-100nM staurosporine in starvation medium initially slightly decelerate their swimming speed (Table 1). At about 1h, they gradually round up and swim more slowly (unpublished experiments).

Apart from proteose peptone (PP), a number of defined peptide hormone chemoattractants may be applied (Köhidai et al. 1994, Leick et al. 1996). One of them is insulin that is a chemoattractant of medium strength. It is active in the concentration range 50 pM to 1μM as shown in Fig. 4A. When using this defined chemoattractant in the two-phase assay the effects of both genistein and daidzein were similar (Fig. 4B) to the effect observed with PP-stimulated chemoattraction.

We also tested the doses/response effect of two different tyrphostins (A47 and A63) on the chemoattraction induced by PP in the two-phase assay as depicted in Fig. 5. It is observed that A47 completely inhibited chemoattraction at 90 μM and only partially at 45 μM. In contrast, A63 a control substance with similar chemical structure but no inhibitory effect (Gazit et al. 1989), did not have any effect at 110 μM but abolished chemoattraction at 480μM. When a series of different A and B tyrphostins was tested it was consistently observed that they had an inhibitory effect on chemoattraction as shown in Table 1. It should be noted, that apart from the negative control substance A63, most of the tyrphostins inhibit at 80-100 μM. Only A48 shows partial inhibition at this concentration. The three B-tyrphostins: B46, B48 and B56 abolish chemoattraction towards proteose peptone at 30 μM. Tyrphostin A25 is a special case where possible effects should be interpreted with caution as it has been reported to be chemically unstable (Ramdas et al. 1994).

When microscopic single cell analysis of treated cells was carried out it showed that the impact of the kinase inhibitors is a desensitisation of the individual cell with respect to its ability to adapt (and respond) to a gradient

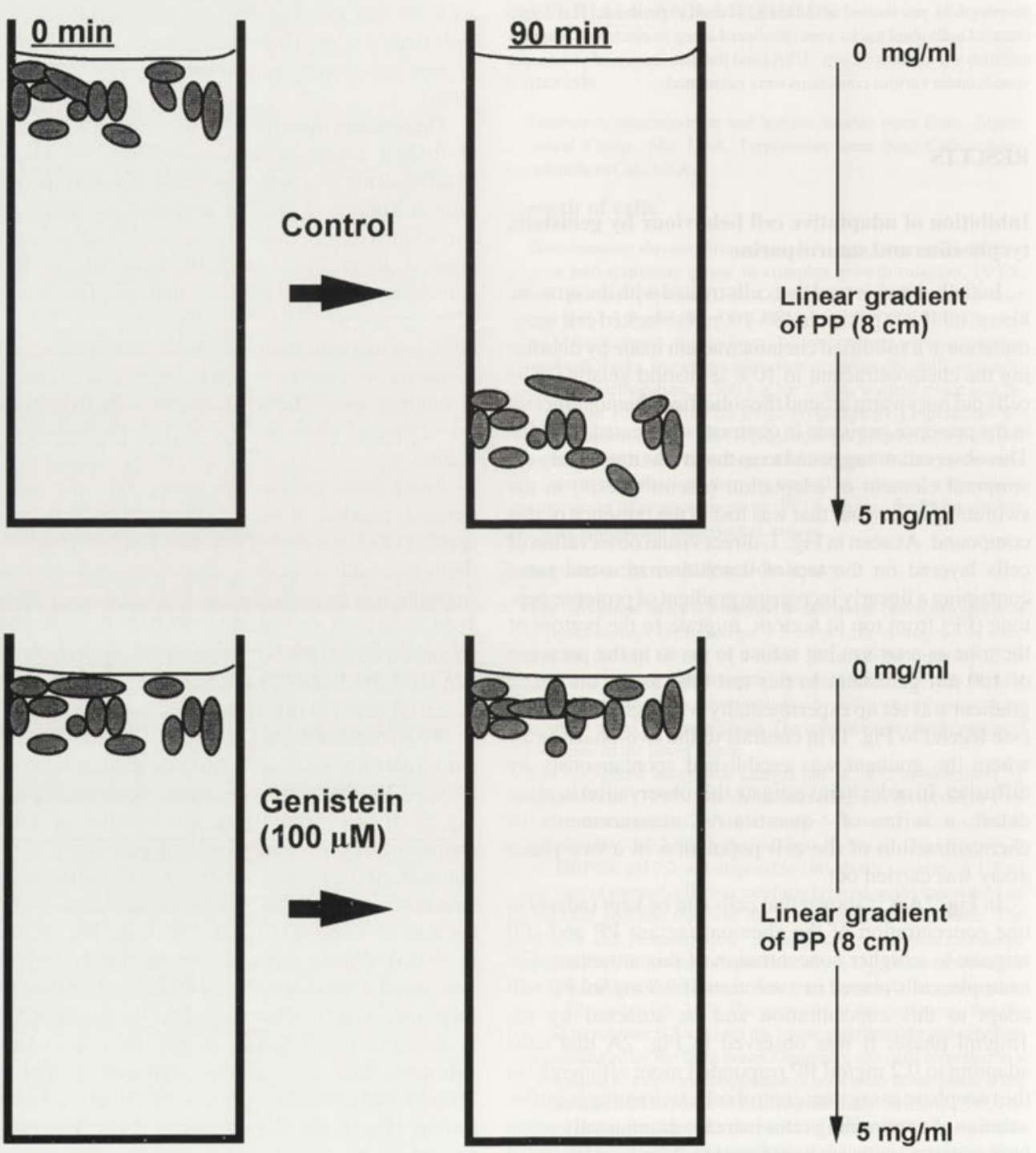


Fig. 1. Schematic drawing of the inhibition by genistein of cellular migration along a proteose peptone (PP) gradient in a test tube set-up. Using 10mM HEPES-buffer containing 1% (w/v) of gelatine a linear gradient of proteose peptone (0 mg/ml at the top to 5mg/ml at the bottom) was established. The 10 ml linear 8 cm long gradient was made in a gradient mixer (also applied for making sucrose gradients for ultracentrifugation) by gradual mixing of 5ml 1% gelatin in HEPES-buffer and 5ml PP (5mg/ml). One% gelatin was added to both solutions in order to stabilise the gradient. The experimental test tubes also contained 100μM genistein unformily present throughout the test tube. 200 μl of a starved cell suspension (10^6 cells /ml) were carefully layered on the top of each test tube and the migration of cells (swarming) was followed visually for 1.5 h at room temperature and scored as indicated in the Figure

Table 1. Effects of protein kinase inhibitors on swimming speeds and on chemoattraction. Swimming speeds were measured (5 min after addition of the drug) by video microscopy as described in Materials and Methods. The swimming speeds (mm/s) indicated are mean values. Protein kinase inhibitor-induced increases in swimming speeds were calculated in % of the unstimulated controls for p-values <0.01. The inhibition of chemoattraction by the same inhibitor concentrations were measured by the two-phase assay

Inhibitor	μM	Swim. speed mm per sec s ± s.d. (no. of cells)	Stimulation in % (p)	Inhibition of chemoattraction (two-phase assay)
Genistein	0	0.15 ± 0.09 (12)		
	37	0.39 ± 0.06 (12)	260 (0.0)	no
	100	0.35 ± 0.05 (12)	230 (0.0)	yes
Daidzein	0	0.09 ± 0.03 (12)		
	118	0.18 ± 0.13 (12)	(0.05)	no
	350	0.22 ± 0.07 (12)	240 (0.0001)	yes
T A1	0	0.19 ± 0.07 (22)		
	10	0.17 ± 0.06 (10)	(0.517)	no
	100	0.28 ± 0.09 (11)	150 (0.002)	yes
T A25	0	0.19 ± 0.09 (23)		
	10	0.18 ± 0.06 (12)	(0.697)	no
	110	0.28 ± 0.1 (11)	(0.061)	no (T A25 is unstable)
T A47	0	0.13 ± 0.06 (24)		
	45	0.28 ± 0.06 (11)	220 (0.0)	no
	91	0.20 ± 0.06 (11)	150 (0.008)	yes
T A63	0	0.1 ± 0.03 (23)		
	116	0.20 ± 0.12 (12)	(0.0687)	no
	480	0.45 ± 0.08 (12)	450 (0.0)	yes
T B42	0	0.11 ± 0.04 (23)		
	30	0.24 ± 0.05 (11)	220 (0.0)	no
	100	0.25 ± 0.03 (12)	230 (0.002)	yes
T B44	0	0.10 ± 0.04 (24)		
	10	0.20 ± 0.13 (12)	(0.034)	no
	100	0.34 ± 0.05 (12)	340 (0.0)	yes
T B46	0	0.10 ± 0.04 (24)		
	10	0.39 ± 0.05 (12)	390 (0.0)	no
	30	0.27 ± 0.04 (12)	270 (0.0)	yes
T B48	0	0.14 ± 0.06 (24)		
	3	0.19 ± 0.09 (13)	(0.134)	no
	30	0.30 ± 0.03 (12)	210 (0.0)	yes
T B50	0	0.08 ± 0.03 (24)		
	30	0.32 ± 0.04 (11)	400 (0.0)	no
	100	0.28 ± 0.04 (13)	250 (0.0)	yes
T B56	0	0.1 ± 0.05 (24)		
	3	0.13 ± 0.06 (13)	(0.169)	no
	30	0.19 ± 0.05 (12)	190 (0.0003)	yes
Staurosporine	0	0.2 ± 0.09 (16)		
	0.05	0.12 ± 0.06 (16)		no
	0.1	0.12 ± 0.05 (16)		yes

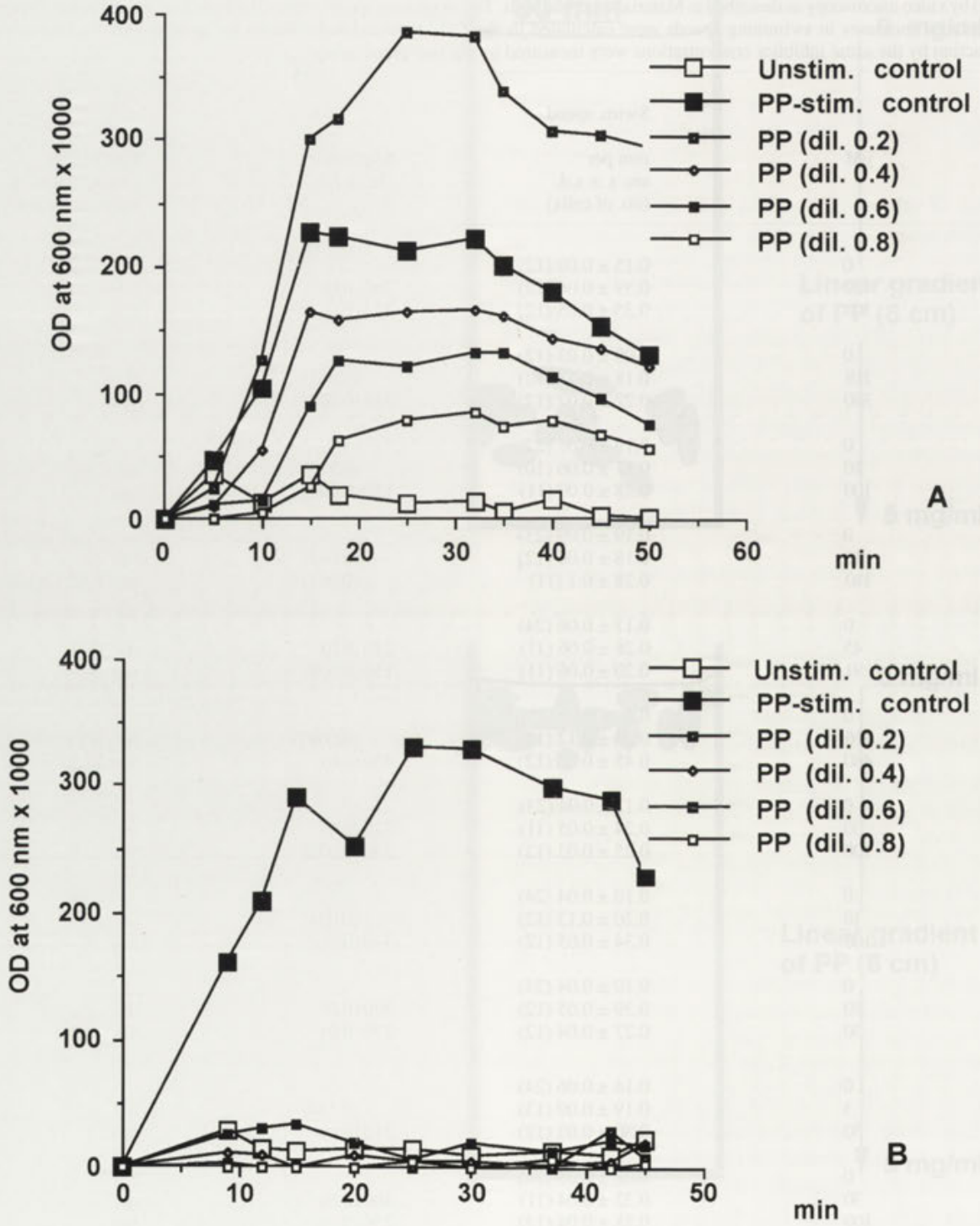


Fig. 2 A - chemoattraction in the two-phase assay of cells adapted to various concentrations of PP. Starved cells preincubated with increasing concentrations of proteose peptone for 5 min (0.2, 0.4, 0.6 and 0.8 mg/ml, respectively) were layered on the top of the metrizamide phase containing 1 mg/ml PP. The appearance of cells in the metrizamide solution containing 1 mg/ml PP (lower phase) was monitored automatically at 600 nm in a recording spectrophotometer at 28°C. 2 B - chemorepulsion from HEPES-buffer of cells adapted to different concentrations of proteose peptone. Cells were pre-treated as in A and layered carefully on the top of the metrizamide phase (no attractant). The PP-stim. chemoattraction control of starved cells was in panel A. The unstimulated controls and the stimulated controls are shown with enlarged symbols

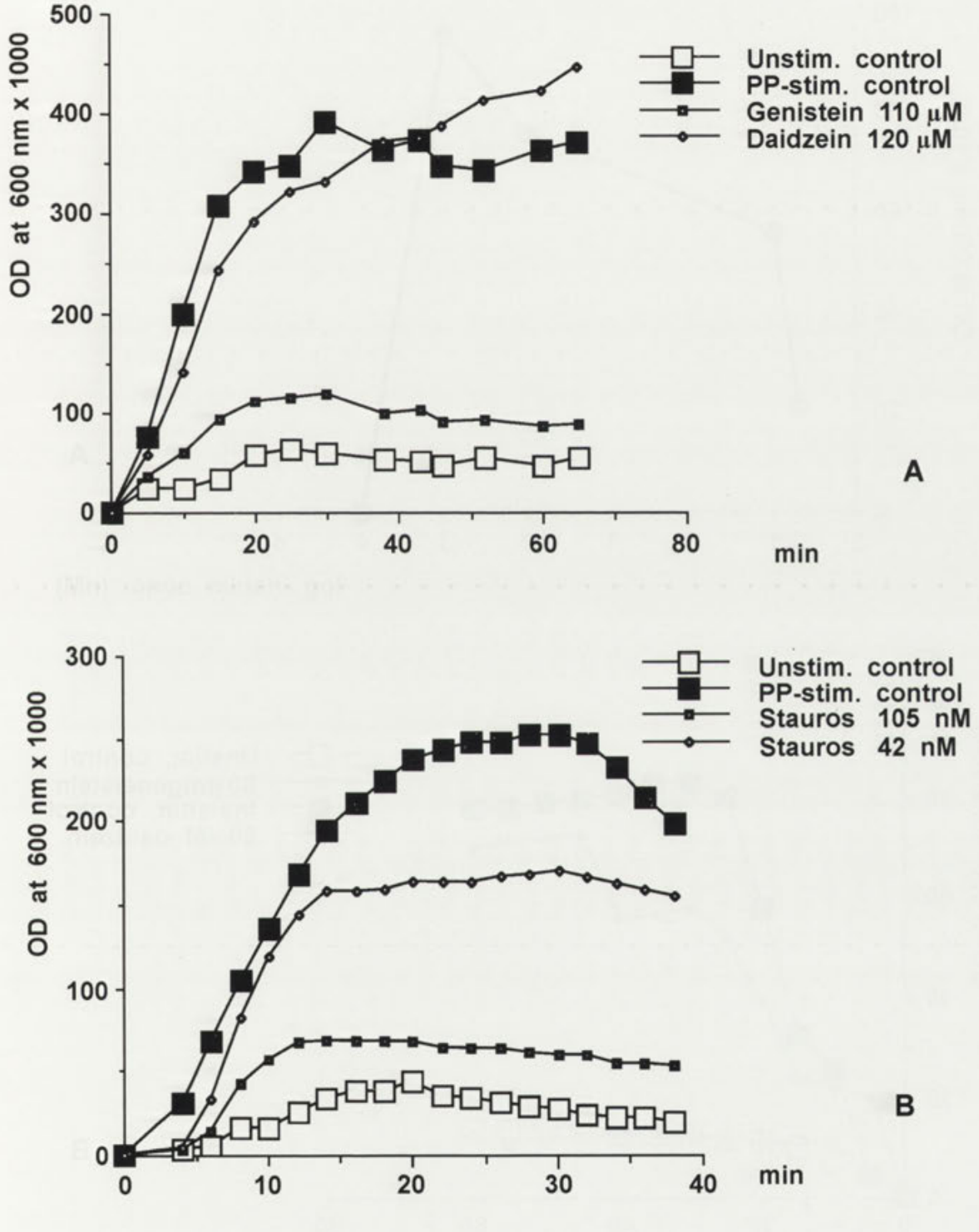


Fig. 3 A - effect of genistein (110 μM) and daidzein (120 μM) on chemoattraction towards PP in the two-phase assay. The assay was carried out in the standard way as in Fig. 2A. Genistein and daidzein were present in both upper and lower phase. B - effect of two different concentrations of staurosporine on chemoattraction towards PP in the two-phase assay. The unstimulated controls and the stimulated controls are shown with enlarged symbols. Inhibitor was present in both upper and lower metrizamide-phase

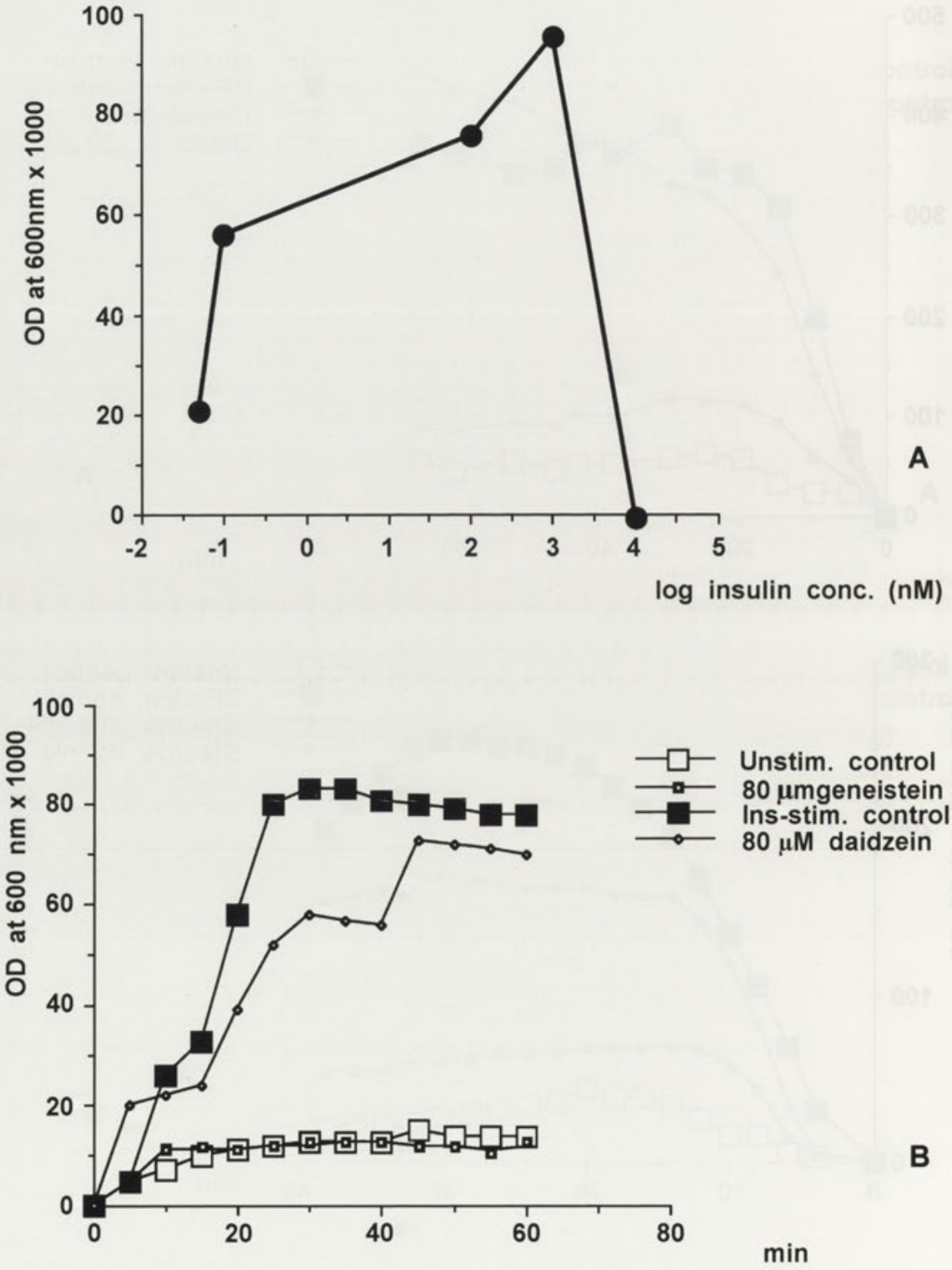
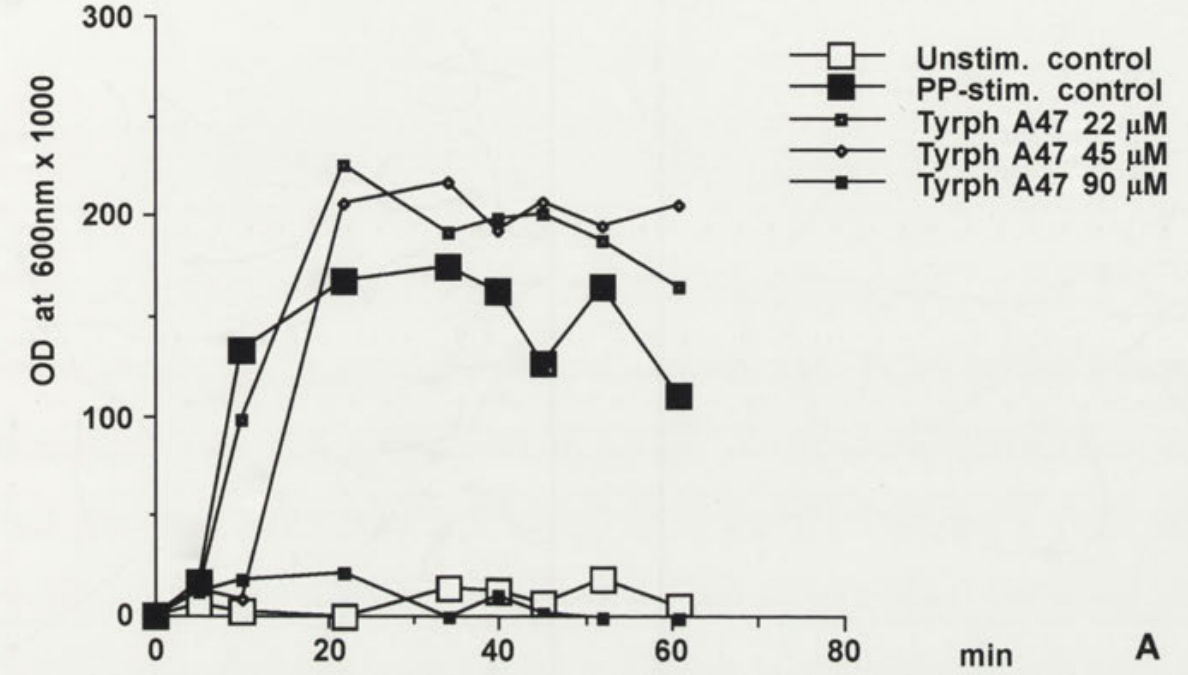


Fig. 4 A - doses/response curve for insulin as chemoattractant in the two-phase assay; number of cells (cell conc.) attracted as a function of insulin concentration in a constant time (45 min). B - time-course of the effect of genistein and daidzein using insulin as chemoattractant. The assay was carried out in the standard way using insulin (100nM) as attractant. Protein kinase inhibitor (80 µM) was present in both upper and lower phase



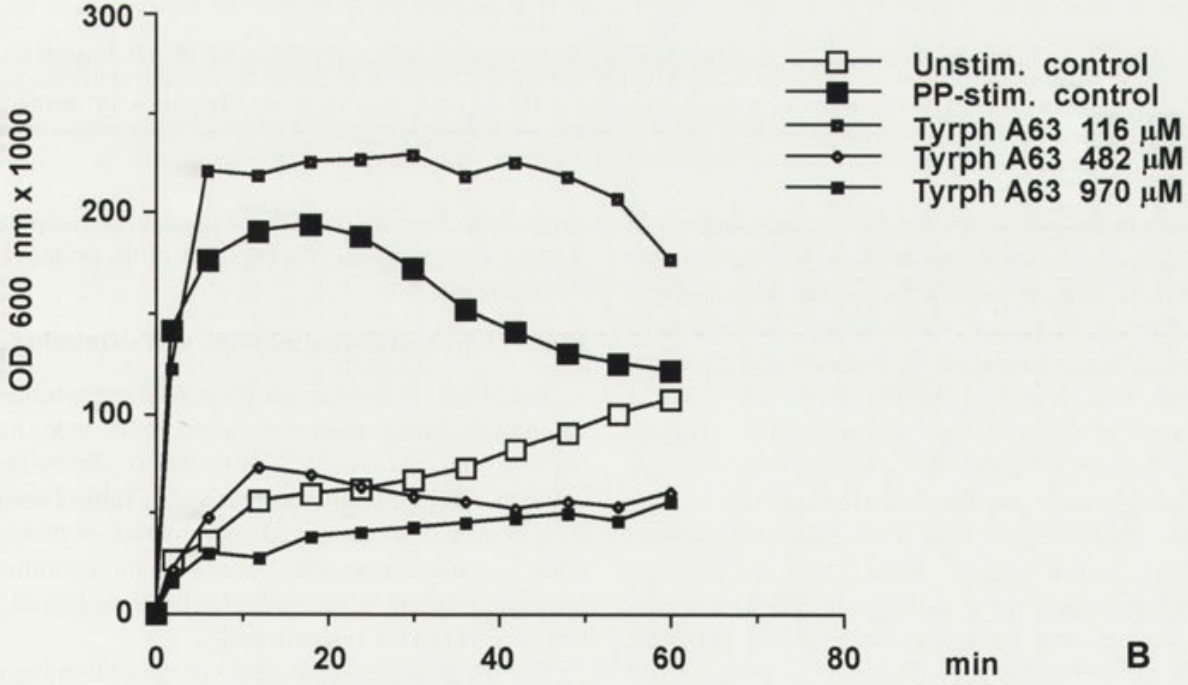


Fig. 5. Doses/response curves of the effects of two different tyrphostins A47 (A) and A63 (B) in the two-phase assay. The assay was carried out in the standard way as described in Fig. 2A (time-course) using PP as attractant. Protein kinase inhibitors were present in both upper and lower phase

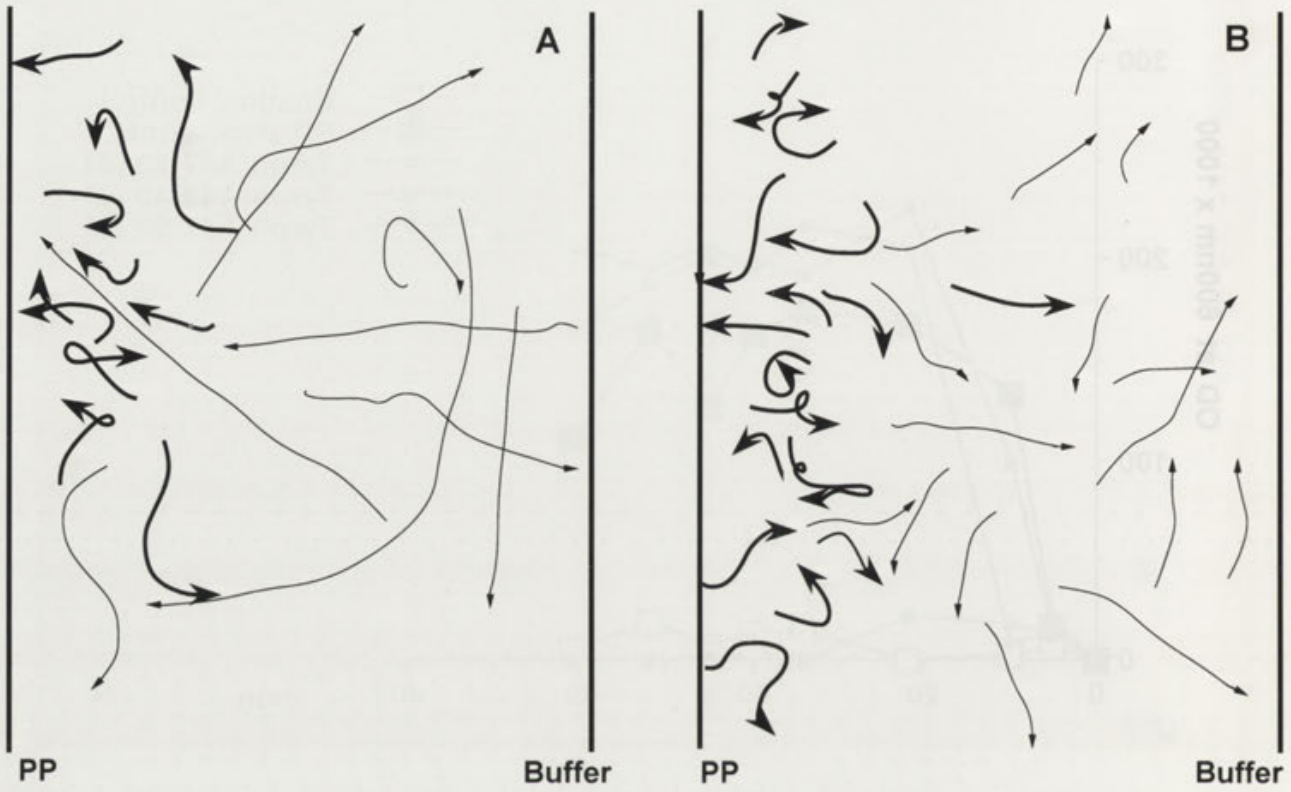


Fig. 6. Change of single cell adaptative behaviour in the Zigmond chamber induced by genistein (A) and tyrphostin A47 (B). PP (1mg/ml) was added to a suspension of starved cells and the suspension was placed in the left well. HEPES-buffer was placed on the right side of the bridge and 2 s (control) swimming tracks of about 10 cells or more were monitored on the 1mm bridge (thick lines). Genistein or tyrphostin A47 (both at 90 μM) was added to both wells and about 10 swimming tracks were measured 10 min after addition of the drug (thin lines). Arrows indicate direction of swimming

of a chemoattractant as shown in Fig. 6. Single cell swimming tracks in the Zigmond chemotactic chamber and the effects of genistein and tyrphostin A47 on single cell swimming behaviour is shown here. Starved cells were placed (and adapting to PP, 1mg/ml) in the well on the left side of the bridge and HEPES-buffer solution was then placed on the right side establishing a diffusion gradient of PP on the bridge. Swimming tracks (duration 2 s) of single cells on the 1mm bridge where they encounter a decreasing concentration of attractant showed that cells were repelled in the control experiment and were prevented from swimming into the HEPES-buffer containing well on the right hand side. When genistein (Fig. 6A) or tyrphostin A47 (Fig. 6B) was added and swimming tracks of single cells were then monitored a dramatic change in swimming behaviour was observed. It was seen that cells within 3-5 min stopped being repelled by the decreasing concentration of attractant (they lost their sense of place) and swam in long straight swimming tracks independant of the direction of the track. This suggests that the individual cell is unable to respond to a chemotactic gradient in the presence of the protein kinase inhibitors used.

Effect of protein kinase inhibitors on swimming speed

The effects of the various protein kinase inhibitors on cellular swimming speed were monitored by video microscopy of individual cells. The results of the swimming behaviour in liquids are summarised in Table 1 and show that, in almost all cases and combination of these compounds, a stimulatory effect was seen on the immediate swimming speeds of individual cells as measured 5 min after the addition to control cells.

The stimulation factor (in %) of the swimming speed compared to control cells was calculated in all cases when P-values were <0.01 and it is seen that in cases when an inhibition of the two-phase assay was observed, it was accompanied by an increase in swimming speed. In many cases, when the concentration of the protein kinase inhibitor was not high enough to abolish adaptation

(as measured in the two-phase assay) a stimulation of the response to the proteose peptone attractant was observed. This may be explained by a more efficient migration of the cells towards the PP phase due to higher speeds of swimming induced by the kinase inhibitors present in a concentration too low to affect chemoattraction. In this respect staurosporine represents an exception as no stimulation of swimming velocity was observed. However, the inhibitory effect of staurosporine on the two-phase assay is due to its interference with adaptation as no chemoattraction was seen for an attractant source embedded in a semisolid (unpublished experiments).

DISCUSSION

Protein kinase inhibitors have a preferential inhibitory effect on the adaptative temporal element of swimming of *Tetrahymena thermophila*. This effect can be monitored by direct microscopical observation of single cell behaviour in the Zigmond chamber and in a quantitative two-phase population assay for chemoattraction.

The abolishment of adaptation can be described as a the induction of a chemical desensitisation leading to compulsive forward swimming and to the inability of the cells to respond to a chemical *gradient* of a chemoattractant.

We observed that, in particular, protein tyrosine kinase (PTK) inhibitors have a stimulatory effect on the immediate speed of swimming indicating that the inhibition of chemoattraction (and adaptation) is not due a decrease of cellular motility. We also tested the effect of staurosporine which is one of the most potent protein kinase inhibitors known. Similar to the PTK inhibitors, staurosporine blocked chemoattraction but it did not increase the immediate swimming speed of the cells. Chemotaxis in neutrophils stimulated with interleukin-8 was inhibited by staurosporine due to its interference with desensitisation (adaptation) (Johnston et al. 1994). In vascular smooth muscle cells it was observed that genistein and an erbstatin analogue, methyl 2,5-dihydroxycinnamate, were inhibitory of PDGF-induced chemotaxis with IC 50 of 150 and 5 μM, respectively (Shimokado et al. 1995). In our system, the tyrphostins B46, B48 and B56 were inhibiting chemoattraction completely at 30μM whereas genistein, A47, A1, B42, B44 and B50 were active starting in the 80-100μM range. The concentration of daidzein and A63 and A48 had to be increased up to 300-500 μM in order to show any inhibitory effect, so in that respect they behave similar to what have been observed in tyrosine kinase *in vitro* systems where they have been used as negative control substances for genistein and tyrphostins, respectively. Tyrphostin A25 seems to represent a special case as it is chemically unstable (Ramdas et al. 1994)) and, accordingly, any results obtained with this compound should be interpreted with caution. It should also be noted that when tyrosine kinase inhibitors were present at low concentration in the two-phase assay an enhancement of the chemosensory response was observed. However, as most PTK-inhibitors increase the swimming speed at these lower concentrations (Table 1) they cause the cells to swim more rapidly into the lower attractant-containing phase at concentrations when adaptation is unaffected, very similar to the situation when adding the chemoattractant PP (0.2 mg/ml) to cells in the upper phase in the two-phase assay (Fig. 2A).

Recently, it was shown that staurosporine inhibits *in vitro* phosphorylation of myelin basic protein fragment 4-14 and phorbol ester-induced *in vivo* phosphorylation in *Tetrahymena thermophila* (Straarup et al. 1997). We have also carried out experiments to elucidate the putative phosphorylation of protein(s) in the presence and absence of PTK inhibitors (Christensen and Leick unpublished). Both *in vivo* labeling with ^{32}P-phosphate and γ-^{32}P-ATP *in vitro* labeling of isolated cilia showed a labeling of distinct proteins. The labeling of some of these proteins are sensitive to the protein kinase inhibitors used in the present study supporting the idea that protein kinase activity is necessary for adaptation leading to stop of ciliary beating or to ciliary reversal. It would be of interest to see how these phosphorylations relate to the control of ciliary beat frequency controlled by cAMP-dependent phosphorylation of a 29 kDa polypeptide in *Paramecium* (Satir et al. 1993).

Acknowledgements. This work was supported by Carlsbergfondet and the Danish Natural Science Research Council. A preliminary report of this work was given at the Meeting of Scandinavian Section of the Society of Protistologists in Helsingør, Denmark, 1997.

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Received on 13th March, 1997; accepted on 8th September, 1997

Acta Protozoologica (1997) 36: 261 - 271

ACTA
PROTOZOOLOGICA

New Thermophilic *Naegleria* Species (Heterolobosea: Vahlkampfiidae) from Australia and Asia: Allozyme, Morphometric and Physiological Characterisation

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Summary. Four new thermophilic *Naegleria* species, capable of growing at 44°C, have been recognised in field studies in Australia, southern Asia and the Pacific. Analysis of allozymes at 32 loci and SSUrDNA sequence data show that they are not closely related to *N. fowleri* or *N. lovaniensis*, from which they can be differentiated using zymograms for esterase, fructose di-phosphatase, glucose-phosphate isomerase and leucine aminopeptidase. The level of allozyme polymorphism within, and the genetic distance between, the new species is consistent with previously described species in this genus. The new species are non-pathogenic to laboratory mice and have not colonised artificial environments as have *N. fowleri* and *N. lovaniensis*. *Naegleria carteri* sp. n. occurs in tropical Australia, in northern tributaries of the Murray-Darling Basin and in Sri Lanka. *N. morganensis* sp. n. has been detected only in the Murray-Darling Basin, while *N. sturti* sp. n. is known from the Murray-Darling Basin and from Bangladesh, Bali and Sri Lanka. *N. niuginiensis* sp. n. is known from a single isolate from Niugini (New Guinea).

Key words: allozyme, biogeography, electrophoresis, *N. carteri, N. morganensis, N. niuginiensis, N. sturti.*

INTRODUCTION

The study of thermophilic *Naegleria* species has evolved from the need to recognise the human pathogen *N. fowleri*, and to distinguish it from other species, both virulent and avirulent. The thermophilic taxa *N. fowleri, N. lovaniensis, N. australiensis, N. italica* and *N. jamiesoni* are now soundly established, but the recognition of new species has some times been a protracted process. For example, organisms now placed in *N. lovaniensis* were long regarded as avirulent strains of *N. fowleri* (De Jonckheere and Van De Voorde 1977).

Recent allozyme studies, employing thirty or more loci to give fine resolution of genetic distance, have revealed the genetic structure of the genus *Naegleria* to be a series of clusters which can be regarded as separate and distinct species (Adams et al. 1989, Robinson et al. 1992). Since all of the named species except the heterogeneous *N. gruberi* correspond to such clusters, this approach permits new genetic groups to be recognised quickly and their rank to be justified rationally. Once a suitable framework is established by identifying the enzymes which have fixed allelic states or limited variation, isozyme techniques using as few as three loci can been used to distinguish *N. fowleri* from *N. lovaniensis* and other *Naegleria* species (Adams et al. 1989, Robinson et al. 1992). Genetic distances calculated from various analyses of nucleic acid variation and the distribution of introns in

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ribosomal DNA confirm the distinctiveness and rank of the well-defined species, as well as the heterogeneity of *N. gruberi* (De Jonckheere 1987, 1988, 1993, 1994a,b; Clark et al. 1989; De Jonckheere and Brown 1994a,b; 1997).

During the course of extensive field studies in Australia and the examination of samples from Asia and the Pacific regions, we have collected a number of thermophilic *Naegleria* isolates that cannot be assigned to described species. Nucleic acid data, recently published for selected isolates from this set, confirm their distinctiveness (De Jonckheere and Brown 1997). This paper reports an allozyme analysis of 18 of these isolates. Diagnoses of four new non-pathogenic species are presented, based on an integration of allozyme, molecular, pathogenicity and morphometric data. The geographic distribution of the new species is discussed.

MATERIALS AND METHODS

Origin of isolates and cultivation

The 24 isolates used in this study, selected from the culture collection of the Australian Water Quality Centre (AWQC), include 18 isolates able to grow at 44°C or higher which could not be placed in any of the described species, and reference isolates of the named thermophilic species, *N. fowleri, N. lovaniensis, N. australiensis, N. italica,* and *N. jamiesoni* (Table 1). *N. australiensis* was represented by two sub groups identified in an earlier study (Adams et al. 1989). Reference strains of the new taxa will be lodged with the American Type Culture Collection (ATCC).

All isolates were grown on 90 mm Petri dishes containing non-nutrient agar spread with live *Escherichia coli* (NNA/*E. coli*) and incubated at 37°C. Enzyme extracts were prepared as previously described (Adams et al. 1989, Robinson et al. 1992).

Allozyme electrophoresis

The enzyme extracts were run on Cellogel Electrophoresis Medium (Chemtron, Milan Italy). The 28 enzymes examined, encoded by 32 putative loci, were those which gave sound results for *Naegleria* species in previous studies (Adams et al. 1989, Robinson et al. 1992), with Gpt and Tpi omitted because reproducible staining was difficult. Electrophoresis and staining conditions were as reported in Table 2 of (Robinson et al. 1992), which also includes lists of the abbreviations and enzyme classification numbers; the genetic data were interpreted as described in that study.

Temperature tolerance

The maximum temperature limit for growth was determined by culturing trophozoites on 13 mm Petri dishes (NNA/*E. coli*) and incubating at 1°C intervals between 42 and 47°C. Isolates were considered to have grown at that temperature if the growth front progressed at least 10 mm from the inoculum.

Growth in axenic culture

A single attempt was made with each isolate to establish axenic growth. Isolates were washed by centrifugation (to remove most bacteria) and inoculated into 50 ml tissue culture flasks containing either 10 ml of Changs Medium (De Jonckheere 1977) or 10 ml of PYNFH Medium (peptone yeast extract, yeast nucleic acid, folic acid and hemin medium), (Laverde and Brent 1980) with the addition of 10% foetal calf serum. The flasks were incubated at 37°C, and examined for growth daily for ten days.

Pathogenicity tests

Pathogenicity was tested in mice for the eighteen isolates not belonging to the described species. *N. fowleri* NG038 was used as a positive and *N. lovaniensis* NG034 as a negative control. Trophozoites were harvested in log phase from 90 mm Petri dishes (NNA /*E. coli*), washed three times in sterile 1/4 strength Ringer solution and diluted to obtain 10^3 cells per 50 µl. Three mice were challenged intranasally with each isolate and monitored for 30 days for mortality.

Microscopy and measurement

Cysts of most strains were examined using an Olympus BH2 microscope and the mean number of pores recorded (n = 50). Measurements of cyst diameter were made by Video Image Analysis (n = 50), as previously described (Dobson et al. 1993).

RESULTS

Allozyme electrophoresis

The 18 thermophilic isolates comprised 16 zymodemes (allelic profiles presented in Table 2). Most isolates were heterozygous at one or more loci, with only two isolates homozygous at all loci tested. The data were converted to a matrix of percent fixed differences which was used in turn to construct a dendrogram (Fig. 1) by the Average Linkage method (Sneath and Sokal 1978). The dendrogram consists of eleven clusters (with some "clusters" consisting of a single isolate).

The isolates representing the described thermophilic species (*N. fowleri*, *N. lovaniensis*, *N. jamiesoni*, *N. australiensis* and *N. italica*) did not cluster with any new isolate. *N. fowleri* was separated from *N. lovaniensis* by more than 60% fixed difference and both were separated from any other cluster by more than 90%. The *N. jamiesoni* isolate (NG157) was separated from the other clusters by an average of 64%. *N. italica* was separated from the other clusters by 73.6% and from the two *N. australiensis* isolates by 89.6%.

Eighteen isolates from undescribed species (sixteen zymodemes) produced five clusters. One of these was formed by a single isolate (NG427) which was separated

Table 1. Nomenclature and origin of *Naegleria* isolates used in this study

AWQC[a] No.	Species	Temp. tolerance	Origin	Cross reference
NG055	*N. carteri* sp. n.	45	sediment, Balonne River, Qld, Aust.	
NG056	" "	45	sediment, Moonie River, Qld, Aust.	
NG057	" "	45	sediment, Moonie River, Qld, Aust.	
NG058	" "	45	sediment, Macintyre River, NSW, Aust.	
NG339	" "	45	sediment, Leichardt Billabong, NT, Aust.	
NG340	" "	45	mine tailings pond, Jabiru, NT, Aust.	
NG341	" "	45	sediment, Koolpin Creek, NT, Aust.	
NG342	" "	45	sediment, East Alligator River, NT, Aust.	
NG346	" "	45	sediment, Woolshed Ck at Surat, Qld, Aust.	
NG221	*N. sturti* sp. n.	44	freshwater, River Murray, S. Aust.	
NG277	" "	44	freshwater, River Lachlan, N.S.W., Aust.	
NG334	" "	44	sediment, L. Keepit, NSW, Aust.	
NG390	" "	44	sediment, Karnafuli R., Bangladesh	
NG236	*N. morganensis* sp. n.	44	freshwater, River Murray, S. Aust.	
NG237	" "	44	freshwater, River Murray, S. Aust.	
NG258	" "	44	freshwater, River Murray, S. Aust.	
NG261	" "	44	freshwater, River Murray, S. Aust.	
NG427	*N. niuginiensis* sp. n.	45	sediment, small lake, Madang, New Guinea	
NG038	*N. fowleri*	45	clinical, S. Aust	ATCC 30214
NG034	*N. lovaniensis*	45	aquarium, Belgium	Aq/1/9/45D
NG035	*N.australiensis*	45	storm water, S. Aust	ATCC 30958
NG202	" "	45	spa pool, S. Aust	ATCC 30958
NG157	*N. jamiesoni*	39	aquarium water, Malawi	Aq/7/S

[a]Formerly SWL No (Adams et al. 1989, Robinson et al. 1992)

from the others by at least 58% fixed difference. The largest cluster of nine isolates, each with a distinct allelic profile (NG055, NG056, NG057, NG058, NG039, NG340, NG341, NG342 and NG346), showed variation at six loci and fixed heterozygosity at Pep3 and Ugpp. The maximum pairwise difference within the cluster was 6.4% and the average distance from the other clusters 64%. Two isolates (NG334 and NG390), which were genetically distinct but shared allelles at all loci (ie. no fixed differences), were separated from the zymodemes represented by NG221 and NG277 at an intermediate level (34% fixed differences) and from the other clusters by 73.6% fixed differences. The final cluster, containing four isolates in two zymodemes (NG236/NG237 and NG258/NG261, represented in Table 2 and Fig. 1 by the first-named strain in each case), also shared allelles at all loci and was separated from the other clusters by an average 57.6%.

Temperature tolerance

The temperature tolerance for each of the isolates is presented in Table 1. The upper limit for growth for *N. fowleri* is between 44 and 46°C (mean 45.0°C), and for *N. lovaniensis* is between 45 and 46°C (mean 45.6°C, unpublished data). The upper limit for growth for the undescribed thermophilic isolates was between 44 and 46°C. The largest group (NG055 etc) was between 44 and 46°C-tolerant (mean 45°C); NG427 grew at 45°C, but none of the other isolates grew above 44°C. Neither *N. australiensis* or *N. italica* grew at 44°C. *N. jamiesoni* NG157, which has been maintained in prolonged axenic culture, was reported originally to be 42°C-tolerant (De Jonckheere 1988), but grew poorly above 39°C in this laboratory and failed to encyst reproducibly. Other environmental isolates of *N. jamiesoni* from Australia have grown at temperatures between 42 and 44°C (BR and PD, unpublished).

Growth in axenic culture

Naegleria fowleri grew in Changs and PYNFH media at 37°C. *N. lovaniensis* grew in PYNFH medium at 37°C but failed to grow in Changs medium after nine days at 37°C. None of the other thermophilic isolates grew in

Table 2. Allelic profiles of thermophilic *Naegleria* isolates

Z'deme	Acn1	Acn2	Adh	Ak1	Ak2	Ald	Acp	Est2	Fdp	Fum	Gapd	Gdh	Got1	Got2	Gpi	Gpt
NG055	aa	aa	cc	bb	cc	—	aa	bb	cc	—	ee	aa	bb	cc	bb	—
NG056	aa	aa	cc	bb	cc	—	aa	bb	cc	—	ee	aa	bb	cc	bb	—
NG057	aa	aa	cc	bb	cc	—	aa	bb	cc	—	ee	aa	bb	cc	bb	—
NG058	aa	aa	cc	bb	cc	—	aa	bb	cc	—	ee	aa	bb	cc	bb	—
NG339	aa	aa	cc	bb	cc	—	aa	bb	cc	—	bb	aa	bb	cc	bb	—
NG340	aa	aa	cc	bb	cc	—	aa	bb	cc	—	dd	aa	bb	cc	bb	—
NG341	aa	aa	cc	bb	cc	—	aa	bb	cc	—	dd	aa	bb	cc	bb	—
NG342	aa	aa	cc	bb	cc	—	aa	bb	cc	—	bb	aa	bb	cc	bb	—
NG346	aa	aa	cc	bb	cc	—	aa	bb	cc	—	ee	aa	bb	cc	bb	—
NG221	cc	ff	dd	bb	cc	dd	ff	ff	ee	aa	bb	aa	cc	dd	cc	cc
NG277	cc	dd	dd	—	cc	—	ff	ff	ee	—	bb	aa	cc	dd	cc	—
NG334	bb	cc	cc	—	cc	—	ff	dd	ee	—	bb	aa	cc	ff	cc	—
NG390	bb	cc	cc	—	cc	—	ff	dd	ee	—	bb	aa	cc	ff	cc	—
NG236	dd	dd	cc	bb	bb	cc	ee	ee	dd	aa	dd	aa	dd	dd	dd	cc
NG258	dd	dd	cc	bb	bb	cc	ee	ee	dd	aa	dd	aa	dd	dd	dd	cc
NG427	cc	ff	cc	—	—	—	—	dd	ee	—	bb	cc	dd	dd	aa	—
NG038	aa	bb	bb	aa	aa	aa	ee	aa	ab	cc	aa	cc	bb	ac	aa	cc
NG034	aa	aa	aa	aa	aa	bb	ee	ee	aa	aa	aa	cc	aa	ee	bb	aa
NG035	dd	ee	cc	bb	dd	ff	dd	cc	ff	cc	ff	bb	bb	bb	ff	aa
NG202	dd	ee	ee	cc	dd	gg	cc	bb	gg	aa	ff	bb	bb	bb	ff	aa
NG036	ee	dd	cc	ab	cc	ee	bb	ff	ee	dd	ee	bb	bb	cc	ee	—
NG157	bb	aa	cc	cc	bb	—	aa	cc	cc	bb	cc	aa	bb	ee	ee	bb

Table 2. (con.)

Z'deme	Hbdh	Hex	Idh	Lap1	Mdh	Me	Mpi	Np	Pep1	Pep2	Pep3	Pgd	Pgk	Pgm1	Thrd	Ugpp
NG055	cc	bb	cc	dd	bb	hi	ee	ee	cc	cc	ef	ee	—	hh	dd	de
NG056	cc	bb	cc	de	bb	hh	ee	ee	cc	dd	ef	ee	—	hh	dd	de
NG057	cc	—	cc	de	bb	hh	ee	ee	cc	dd	ef	ee	—	hh	dd	de
NG058	cc	bc	cc	ce	bb	hi	ee	ee	cc	cc	ef	ee	—	hh	dd	de
NG339	cc	bb	cc	cc	bb	hh	ee	ee	cc	cc	ef	ee	—	gh	dd	de
NG340	cc	bb	cc	bc	bb	hh	ee	ee	cc	dd	ef	ee	—	gh	dd	de
NG341	cc	bb	cc	ce	bb	hh	ee	ee	cc	cc	ef	ee	—	gh	dd	de
NG342	cc	bb	cc	cd	bb	hh	ee	ee	cc	cd	ef	ee	—	hh	dd	de
NG346	cc	bb	cc	ce	bb	hi	ee	ee	cc	cc	ef	ee	—	gh	dd	de
NG221	cc	gg	ee	gg	cc	ee	aa	cc	ff	gg	dd	ee	cc	be	cc	dd
NG277	cc	gg	be	gg	cc	be	—	cc	cf	gg	dd	ee	—	be	cc	dd
NG334	bb	ce	ee	hh	cc	be	—	dd	cf	gg	dd	ee	—	bb	cc	cd
NG390	bb	ee	ee	hh	cc	be	—	dd	cf	gg	dd	ee	—	bd	cc	cd
NG236	cc	ff	bb	ee	cc	aa	hh	cc	cc	hh	ff	cc	ee	gg	dd	bc
NG258	cc	ff	bb	ee	cc	aa	hh	cc	cc	hh	ff	cc	ee	gg	dd	cc
NG427	ee	ce	cc	ef	cc	dd	fh	cc	aa	ii	ff	cc	—	cc	dd	cc
NG038	aa	ee	aa	aa	aa	cc	bb	bb	bb	bb	aa	dd	aa	ee	aa	aa
NG034	aa	dd	aa	bb	aa	dd	cc	aa	bb	aa	bb	fg	bb	aa	bb	bb
NG035	dd	ci	ff	ij	dd	gg	gi	gh	ee	ee	ee	dd	ee	ii	ee	fg
NG202	dd	cc	ff	jk	dd	gg	dd	ff	dd	ff	ee	dd	ef	ik	ee	gg
NG036	dd	hh	dd	dd	dd	ff	dd	de	bb	cc	cc	aa	dd	ff	ff	ff
NG157	ee	aa	bb	cd	bb	gg	aa	dd	ee	cc	gg	bb	dd	gj	dd	hh

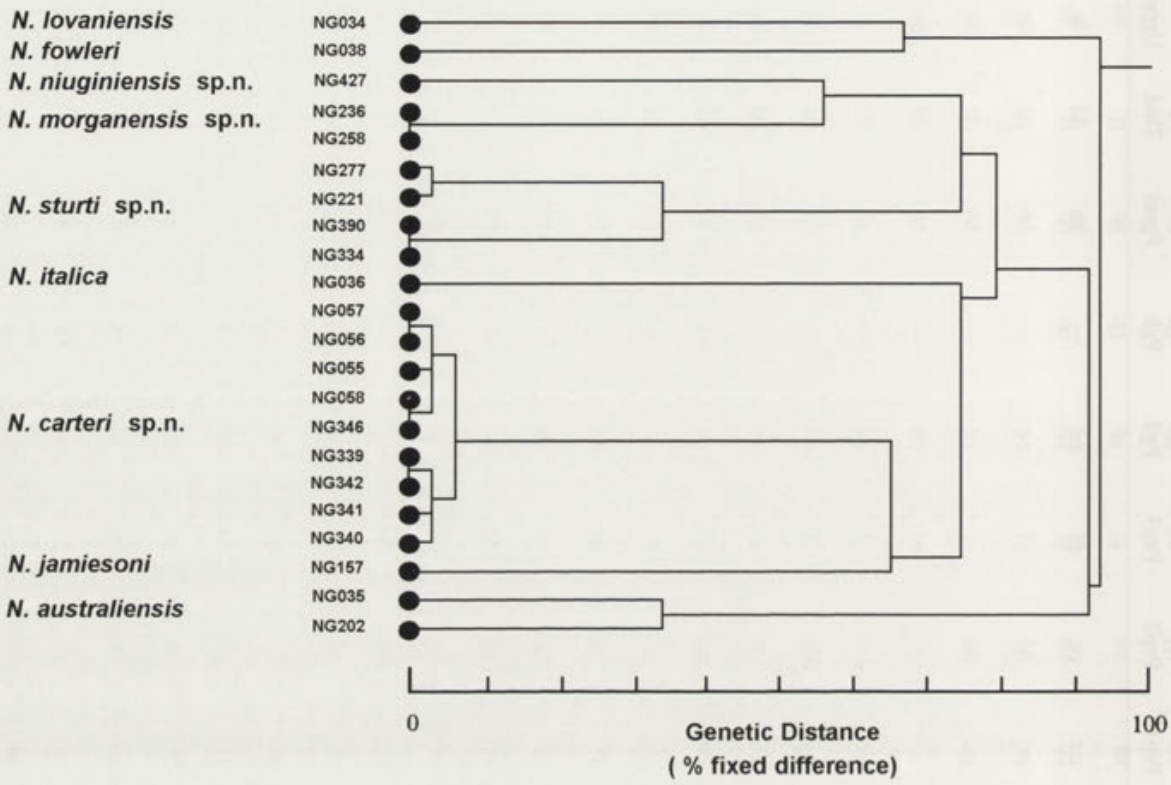

Fig. 1. Relationships among thermophilic *Naegleria* strains, including representative strains of previously described species, calculated by the Average Linkage method (Sneath and Sokal 1978)

either Changs or PYNFH media at this single attempt. It is likely that with persistence most *Naegleria* cultures could be adapted to grow in axenic culture.

Pathogenicity

None of the undescribed isolates were pathogenic to mice after 30 days. All positive control animals (infected with *N. fowleri*) died, while mice infected with *N. lovaniensis* also survived.

Morphological characteristics

Morphometric data are presented in Table 3. Observation of cysts during isolation has shown that some species have distinctive characteristics, such as a loose ectocyst in the isolates NG221, NG277, NG236, NG237, NG258 and NG 261, or a high proportion of vacant cyst walls, as in *N. australiensis* (NG035).

Geographic distribution.

The precise origin of each strain is shown in Table 1, while the geographic distributions of the new *Naegleria* species are illustrated in Fig. 2. The maps include additional data for strains that were isolated after the detailed allozyme analysis was completed, in particular a set of strains from Sri Lanka, and identified by isozyme patterns for the diagnostic loci (see Diagnoses).

DISCUSSION

Recent systematic studies of the genus *Naegleria* have concentrated on genetic analysis to measure relationships, and hence to distribute strains within or among species. Allozyme analysis (i.e. rigorous genetic interpretation of isozyme data) is particularly useful since it can be applied inexpensively to a large number of strains, for a large number of gene loci (Adams et al. 1989, Robinson et al. 1992). Such a data matrix can be used to test whether variation is discontinuous, i.e. whether strains cluster closely, with readily-recognisable boundaries that allow them to be treated as species (Adams et al. 1989, Robinson et al. 1992). If clusters are discrete in this way, interspecific and intraspecific variation are distinguished as a matter of course, permitting a confident choice of

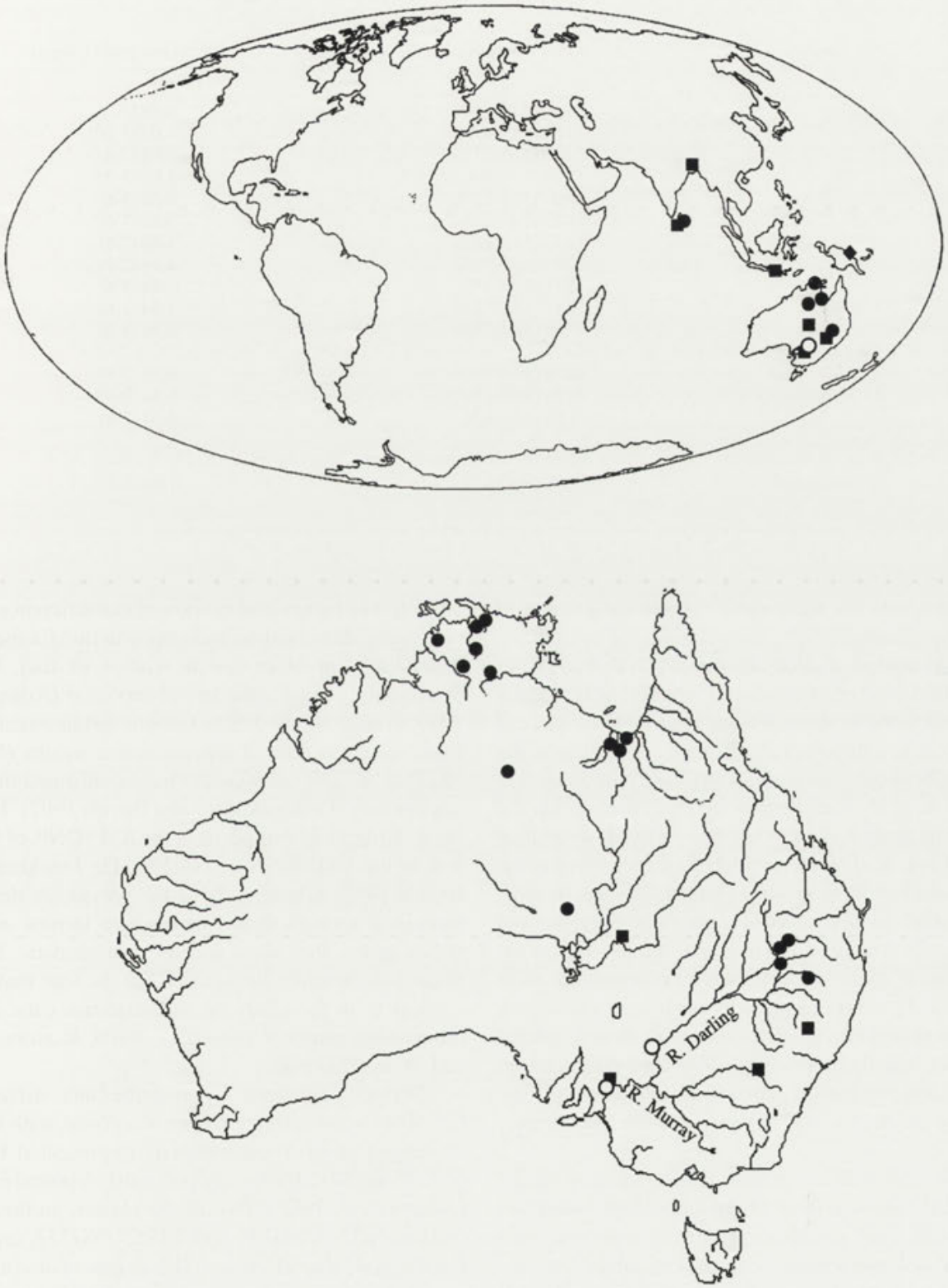

Fig. 2. Known geographic distribution of new thermophilic *Naegleria* species, globally (above) and within Australia (below). ● *N. carteri*, ○ *N. morganensis*, ◆ *N. niuginiensis*, ■ *N. sturti*

Table 3. Morphometric data for new thermophilic *Naegleria* species

AWQC	No. species	Mean cyst dia. (range), μm	Mean no. pores (range)
NG055	*N. carteri*	10.81 (9.5-12.8)	4.88 (3-7)
NG056	" "	14.81 (10.6-19.6)	6.02 (3-10)
NG057	" "	14.70 (12.5-17.6)	5.08 (3-8)
NG058	" "	12.52 (9.7-19.4)	5.08 (3-8)
NG339	" "	12.34 (10.6-15.4)	4.56 (3-8)
NG340	" "	10.27 (8.1-13.0)	3.68 (1-6)
NG341	" "	14.12 (11.7-16.5)	5.86 (3-9)
NG342	" "	12.92 (10.1-21.6)	4.16 (2-7)
NG346	" "	11.11 (9.5-13.0)	4.48 (3-8)
NG390	*N. sturti*	19.11 (12.78-27.8)	3.94 (1-6)
NG334	" "	17.05 (12.1-23.5)	3.24 (1-6)
NG221[a]	" "		
NG277	" "	18.34 (13.6-29.0)	4.16 (2-10)
NG236	*N. morganensis*	19.01 (16.7-24.0)	5.54 (3-9)
NG237	" "	11.82 (9.0-14.5)	3.04 (2-4)
NG258[a]	" "		
NG261[a]	" "		
NG427	*N. nuginiensis*	11.83 (9.0-14.7)	1.94 (1-5)

[a]Isolate not measured

genetic markers for subsequent routine assignment of isolates to species.

A disadvantage of allozyme analysis is that as evolutionary lines diverge, some genetic changes will produce convergent mobility states, leading to an underestimate of divergence. In a dendrogram drawn from such data, the distances between nodes are in any case compressed as the percentage difference approaches 100%, so that the order of branching may be unreliable. Analysis of variation at the nucleic acid level, particularly direct sequencing, provides the best information on longer distances, since it usually targets more conserved regions of the genome and the scale of variation is open-ended. It can confirm the distinctness of species, determine their relationships more precisely and position genera in higher taxa. Because such sequencing is relatively laborious, fewer closely-related strains are usually studied and less data produced on variation and population genetics of individual species. Allozyme and nucleic acid analysis are therefore complementary.

By allozyme analysis, genetic distances among strains of *Naegleria* are distributed bimodally (Robinson et al. 1992), with short distances within clusters and long distances between clusters. The thermophilic isolates studied here form distinct clusters which are as well defined as those corresponding to the described species, differing at more than 50% of loci [Fig. 1, compare dendrograms in Adams et al. (1989) and Robinson et al. (1992)]. The earlier studies showed that differences in this range provide a consistent species criterion for the genus, depending somewhat on the choice of loci, but the discontinuity itself is the key observation (Adams et al. 1989, Robinson et al. 1992). Genetic distances calculated from sequence data of representative strains (NG055, NG334, NG236 and NG427) have confirmed their distinctiveness (De Jonckheere and Brown 1997). Furthermore, Group I introns occur in the SSUrDNA of NG055 and in the LSUrDNA of NG236 (De Jonckheere and Brown 1997). Close study of the previously described *Naegleria* species shows that where introns occur in rDNA genes, they are a species characteristic. Species status can therefore be justified for the four major new clusters from the allozyme and sequence data, and we propose the names *N. carteri, N. sturti, N. morganensis* and *N. niuginiensis*.

Clusters separated by intermediate differences (25-50%) occur infrequently in *Naegleria*, with the pair of subgroups of *N. australiensis* (represented here by NG035 and NG202) the only previously reported example (Adams et al. 1989). Two of the clusters in this study, isolates NG334/NG390 and NG221/NG277, are separated by a similar difference. This degree of discontinuity can be interpreted as incipient speciation, but we are not proposing separate formal names at this time.

Relationships among the existing and the new species are corroborated by the sequence and allozyme analyses.

N. morganensis and *N. niuginiensis* cluster together, then with *N. sturti* (albeit at long genetic distances), while *N. carteri* clusters most closely, among the thermophilic species, with *N. jamiesoni*. *N. minor*, the nearest species to *N. carteri* on the evidence of SSUrDNA sequences (De Jonckheere and Brown 1997), was not included in the present analysis. The agreement of the two approaches suggests that the branching order on the dendrogram calculated from allozyme data is accurate to approximately 70% on the fixed difference scale (see earlier discussion of inter-node distances).

While morphological and physiological characteristics cannot be used alone to determine the identity of a fresh *Naegleria* isolate, knowledge of the source and temperature tolerance combined with, in some cases, specific characteristics of the cyst (loose ectocyst or high proportion of vacant cysts) may be used as a guide for further identification. Cyst characters can also vary with growth conditions; amoebae grown in axenic culture or on moist plates usually produce more regular cyst shapes than those grown under drier conditions. Rapid adaptation to axenic culture, particularly in Chang's Medium, can be a useful preliminary clue to the identity of *N. fowleri* and even a selective step in its isolation from mixed populations (De Jonckheere 1977). However, definitive identification requires molecular or biochemical comparison with a number of reference strains.

Diagnoses

The diagnoses that follow assume the characters that are common to *Naegleria* species at the generic level, i.e. amoeboid, eruptively motile trophozoites; non-trophic, usually non-dividing flagellate stage and excystment *via* preformed pores. The diagnoses incorporate, in concise form, the molecular characters recently published for these organisms (De Jonckheere and Brown 1997).

Naegleria carteri sp. n.

Cyst approximately circular, with closely opposed endocyst and ectocyst walls, maximum dimension 8.1 to 21.6 µm (mean 12.6 µm); 1 to 10 excystment pores (mean 4.6) in mononucleate cysts; all stages predominantly mononucleate. Maximum growth temperature 44-46°C (mean 45°C). Characteristic alleles expressed at Acn1, Acn2, Adh, Ak1, Ak2, Acp, Est2, Fdp, Gdh, Got1, Got2, Gpi, Hbdh, Idh, Mdh, Mpi, Np, Pep1 and Thrd; heterozygosity predominant or fixed for Pep3 and Ugpp. Unique SSUrDNA sequence, including a Group I intron with a sequence divergent from others occurring in the genus.

Reference strain: AWQC NG055.

Intraspecific variation: allelic polymorphisms expressed at Gapd, Hex, Lap1, Me, Pep-2, and Pgm1.

Known geographic distribution: Australia and Sri Lanka.

Naegleria carteri is named in honour of Prof. Rodney Carter, for his contribution in the recognition of the disease primary amoebic meningoencephalitis.

Naegleria sturti sp. n.

Cyst approximately circular, with closely opposed endocyst and ectocyst walls, or more irregular with the ectocyst wall separated from the endocyst wall, maximum dimension 12.1 to 29.0 µm (mean 18.2 µm); 1 to 10 excystment pores (mean 3.8) in mononucleate cysts. All stages predominantly mononucleate. Maximum growth temperature 44°C. Characteristic alleles expressed at Ak2, Acp, Fdp, Gapd, Got1, Gpi, Mdh, Pep2, Pep3, Pgd and Thrd. Unique SSUrDNA sequence. Two subgroups distinguished by allelic states for Acn1, Adh, Est2, Got2, Hbdh, Hex, Lap1, and Np.

Reference strain: AWQC NG334.

Intraspecific variation: allelic polymorphisms expressed for Acn2, Hex, Idh, Me, Pep1, Pgm1 and Ugpp.

Known geographic distribution: Australia, Bangladesh and Sri Lanka. Both sub-groups occurring in Australia and Asia.

Naegleria sturti is named after Charles Sturt, the first European to explore the River Murray, in 1829-30.

Naegleria morganensis sp. n.

Cyst approximately circular, with loosely opposed endocyst and ectocyst walls, maximum dimension 9.0 to 24.0 µm (mean 15.4 µm); 2 to 9 excystment pores (mean 4.3) in mononucleate cysts. All stages predominantly mononucleate. Maximum growth temperature 44°C. Characteristic alleles expressed at Acn1, Acn2, Adh, Ak1, Ak2, Acp, Est2, Fdp, Gdh, Got1, Got2, Gpi, Hbdh, Idh, Mdh, Mpi, Np, Pep1 and Thrd. Unique SSUrDNA sequence; LSUrDNA including a Group I intron; length of ITS region extended by a unique insert.

Reference strain: AWQC NG236.

Intraspecific variation: allelic polymorphisms expressed for Ugpp.

Known geographic distribution: Australia.

Naegleria morganensis is named after the town of Morgan, on the River Murray where it was first isolated.

Naegleria niuginiensis sp. n.

Cyst approximately circular, with closely opposed endocyst and ectocyst walls, maximum dimension 9.0 to

Table 4. Characteristic allelic states for thermophilic *Naegleria* species at diagnostic loci

Species	AWQC No.	Est2	Fdp	Gpi	Lap1
N. fowleri	NG038	a	ab	a	a
N. lovaniensis	NG034	e	a	b	b[a]
N. italica	NG036	f	e	e	d
N. australiensis	NG035	c	f	f	ij[a]
N. australiensis subgp	NG202	b	g	f	jk[a]
N. jamiesoni	NG157	c	c	e	cd
N. carteri	NG055	b	c	b	d[a]
N. sturti	NG221	f	e	c	g
N. sturti subgp	NG334	d	e	c	h
N. morganensis	NG236	e	d	d	e
N. nuginiensis	NG427	d	e	a	ef

[a]Variable for other isolates of this species

14.7 μm (mean 11.8 μm); 1 to 5 excystment pores (mean 1.9) in mononucleate cysts. All stages predominantly mononucleate. Maximum growth temperature 45°C. Characteristic alleles expressed at numerous loci. Unique SSUrDNA sequence.

Reference strain: AWQC NG427.

Intraspecific variation: unknown

Known geographic distribution: Niugini (New Guinea).

Naegleria niuginiensis is named for its geographic origin.

Identification

Because of the increasing number of described *Naegleria* species, it is becoming more important to have a rapid and reliable diagnostic tool, such as allozyme electrophoresis, to distinguish between the pathogenic *N. fowleri* and other species. This study identifies the allozyme loci that exhibit fixed allelic states within species and useful differences among species, from which diagnostic criteria can be chosen. To characteristic 'new' environmental isolates, zymograms for four or five enzymes are usually sufficient for identification and to provide some data on intraspecific variation. The identification scheme used at AWQC to identify thermophilic *Naegleria* species is shown in Table 4. Any *Naegleria* isolate NOT matching a reference strain closely by these criteria is likely to represent a previously unrecognised species.

Ecology and biogeography

Naegleria carteri is widespread in tropical Australia and in northern parts of the Murray-Darling Basin, particularly in certain tributaries of the River Darling. *N. sturti* and *N. morganensis* occur in southern parts of the basin more frequently than *N. fowleri* and at similar frequencies to *N. lovaniensis*. *N. carteri* and *N. sturti* also occur in isolated, sometimes ephemeral, water bodies in arid, inland Australia. None of the new species have been detected in many years monitoring of public water supplies drawn from the River Murray, suggesting that they colonise artificial environments rather poorly. In the same period, *N. fowleri* and *N. lovaniensis* occasionally reached several hundred organisms per litre in water supplies, though they were rarely detected in the natural source waters (BR and PD, unpublished studies).

Our knowledge of the global distribution of the new species is incomplete, but presumably they may be widespread if uncommon in tropical Asia. Whether they occur more widely still in tropical environments is unclear, since very few *Naegleria* isolates have been studied from Africa or Central and South America. The likely distribution of a species is not predictable simply from its temperature requirements, since some *Naegleria* species which occupy almost identical thermal niches (eg *N. australiensis*, *N. italica* and *N. jamiesoni*) have very different distributions.

Significance of heterozygosity

One feature of natural populations of *Naegleria* species that has been discussed recently is their mode of reproduction. In *N. lovaniensis*, the relative frequency of homo- and heterozygotes at several polymorphic loci is reported to conform to Hardy-Weinberg equilibrium, while allelic distribution among multilocus genotypes is strongly suggestive of recombination by sexual reproduction or some equivalent process (Pernin et al. 1992). Among the new species described here, there is enough data to be useful in this discussion only for *N. carteri*. The nine strains studied for this species were universally heterozygous at two loci, a strong indication that growth is exclusively clonal, since sexual reproduction at any frequency would rapidly produce homozygotes through segregation (Tibayrenc et al. 1990). Several mesophilic genetic groups still treated as falling within *N. gruberi* also exhibit predominant or fixed heterozygosity (Robinson et al. 1992). If sexual reproduction does occur in *N. lovaniensis*, it may therefore be exceptional rather than typical of *Naegleria* and this species is worthy of closer study.

Acknowledgments. We thank G. Ganf, P O'Donoghue, J. Puckridge, T. Reardon and J. Shepherd for collecting environmental material. We

are especially grateful to Johan De Jonckheere and Susan Brown for allowing us to read a related manuscript before publication and for helpful comments on the present work.

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Received on 23th June, 1997; accepted on 30th July, 1997

Acta Protozoologica (1997) 36: 273 - 278

ACTA
PROTOZOOLOGICA

Defining New *Naegleria* spp. Using Ribosomal DNA Sequences

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Summary. Part of the small subunit ribosomal DNA (SSUrDNA) and the internal transcribed spacer (ITS) region, including the 5.8SrDNA, of six *Naegleria* strains have been sequenced. The strains are thermophilic and, based on allozyme studies, some of them may be considered to represent new *Naegleria* species. From the combination of SSUrDNA sequence differences, ITS sequence comparisons, and presence or absence of group I introns within the rDNA, it is concluded that all six strains can be considered to be new species. The relationships of these strains to described *Naegleria* spp. are inferred from rDNA sequence comparisons and are presented in a phylogenetic tree.

Key words: 5.8SrDNA, ITS, *Naegleria*, phylogenetic tree, SSUrDNA, thermophilic.

INTRODUCTION

Species identities within the amoeboflagellate genus *Naegleria* were confirmed by comparing a 800 bp sequence between two conserved Pst I sites in the small subunit ribosomal DNA (SSUrDNA) (De Jonckheere 1994a). These two Pst I sites had been found to be characteristic of the genus *Naegleria*. Sequence determination of this part of the SSUrDNA was used subsequently as an important additional criterion for describing new species (De Jonckheere 1994a, Pernin and De Jonckheere 1996). *Willaertia minor* was redefined as a *Naegleria* sp. based on SSUrDNA sequence information (De Jonckheere and Brown 1994a). In addition, the sequence of the SSUrDNA proved the existence or synonymy of some species and genera (De Jonckheere et al. 1996). Allozyme studies have shown that probably many more *Naegleria* spp. than the ones presently described need to be established (Robinson et al. 1992). We have used partial SSUrDNA sequence determination to test this hypothesis on some unusual *Naegleria* strains which, based on allozyme studies, will be described as new species (Dobson et al. 1997) in this issue and on strains that proved to have unique group I introns within the rDNA (De Jonckheere and Brown 1994b, and unpublished). Since no morphological markers are available to differentiate *Naegleria* spp. we propose SSUrDNA sequencing as the preferred method for the description of *Naegleria* spp. This method has the additional advantage that it establishes the phylogenetic relationships between the species. Sequencing the ITS region was performed as well as the ITS has more discriminating power than the

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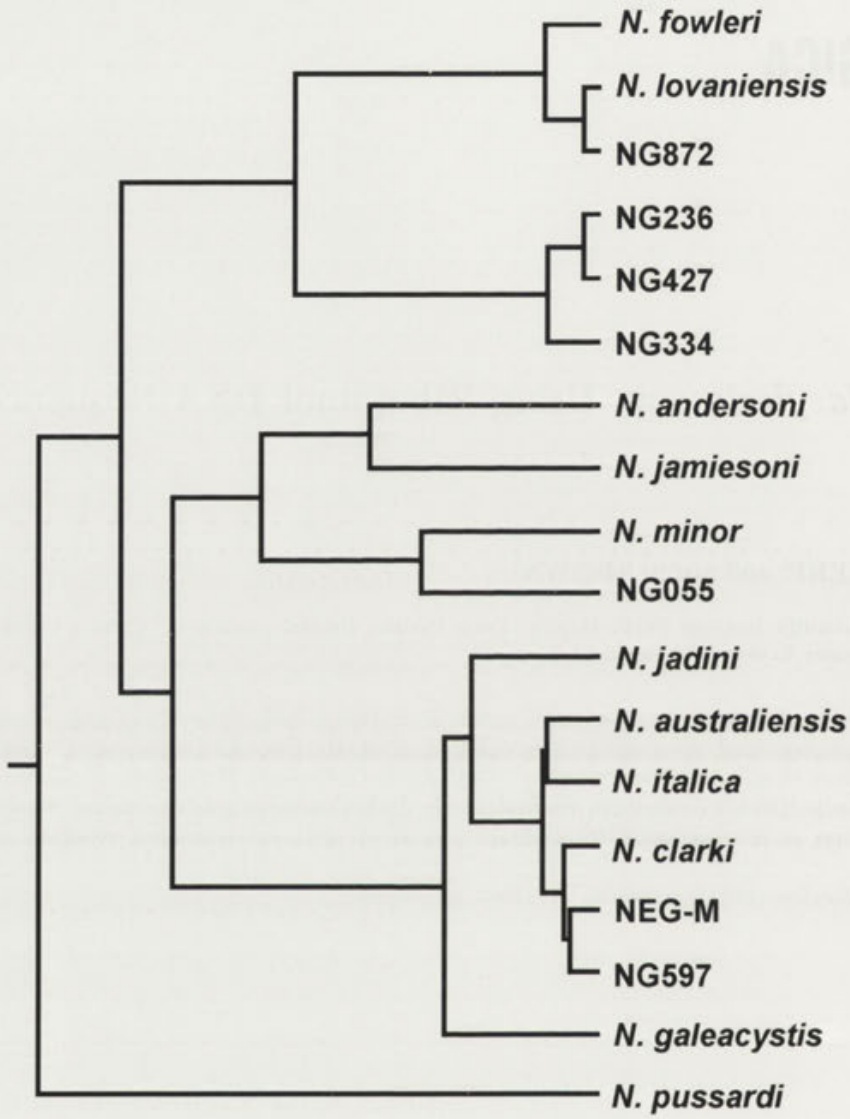

Fig. 1. Phylogenetic tree built with the UPGMA method based on the alignment of partial SSUrDNA sequences (805 bp)

SSUrDNA when comparing closely related strains (De Jonckheere, in press).

MATERIALS AND METHODS

Strains NG055, NG236, NG334 and NG427 are thermophilic *Naegleria* strains which, based on allozyme analysis, are considered to represent new *Naegleria* species (Dobson et al. 1996). A fifth strain, NG 872, was included in the current investigation as it is considered to be a variant of *N. lovaniensis* (Robinson, pers. comm.). These new isolates were obtained from Bret Robinson of the Australian Centre for Water Quality Research in South Australia, who isolated them from the environment in Australia. These five strains can grow at 44°C and are, therefore, called thermophilic. They are non-pathogenic for mice. Only strain NG236 has been included in a previous publication (Adams et al. 1989). Strain NG597 was included as it formed, together with two related strains, a cluster that had lost the open reading frame (ORF) from the group I intron present in the SSUrDNA of several *Naegleria* spp. (De Jonckheere 1993, De Jonckheere and Brown 1994b). Strain NG597 was isolated also in Australia and is 42° C-tolerant (Robinson, pers. comm.). The amoebae were grown on non-nutrient agar plates with *Escherichia coli* and concentrated by centrifugation. The DNA was extracted using a guanidium thiocyanate-Sarkosyl method (Pitcher et al. 1989). The SSUrDNA and the ITS, including the 5.8SrDNA, were amplified using primers and polymerase chain reaction (PCR) conditions described previously (De Jonckheere 1994a and in press). The

PCR products were sequenced using the PCR product sequencing kit (Amersham Life Science Inc., Cleveland, Ohio, USA) using the SSUrDNA amplification primers and conserved internal primers (De Jonckheere 1994a and in press). Reaction products were separated on 6% acrylamide-urea sequencing gels and autoradiographed overnight at room temperature.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers Y10184 till Y10197. The partial SSUrDNA sequences were aligned with those published for described *Naegleria* spp. (De Jonckheere 1994a). Phylogenetic trees were constructed from the aligned sequences using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (Neighbor joining and UPGMA), FITCH (Fitch-Margoliash), KITCH (Fitch-Margoliash with evolutionory clock) and SEQBOOT (bootstrapping) programs of the PHYLIP (version 3.572c) package. Putative secondary structure predictions for the ITS2 sequence of NG236 was obtained using the mfold program (version 2.3) of M. Zucker and D. H. Turner (Walter et al., 1994) for RNA folding by energy minimization.

RESULTS

A longer PCR product (3.3 kb) was obtained from the SSUrDNA of strains NG055 and NG872 than from the three others (2.0 kb) (not shown). This suggests that the SSUrDNA of NG055 and NG872 contains a group I intron, as found in some other *Naegleria* spp. (De Jonckheere 1993). Strain NG597 yielded a PCR product of 2.4 kb as the SSUrDNA group I intron in this strain has lost the ORF (De Jonckheere and Brown 1994b). The sequences between the two Pst I sites were aligned with those of the 12 described *Naegleria* species and these alignments were used to generate phylogenetic trees (Fig.1). Strain NG597 branches within the *N. australiensis* - *N. italica* - *N. clarki* cluster with strain NEG-M of *N. gruberi*. Strain NG872 is situated on the *N. lovaniensis* - *N. fowleri* branch. Strain NG055 bifurcates from the *N. minor* branch, which is itself close to the *N. andersoni* - *N. jamiesoni* branch. The other three strains form a separate cluster close to the *N. lovaniensis*-*N. fowleri* branch.

The SSUrDNA group I intron sequence of NG055 was aligned with those of the introns of other *Naegleria* spp. (De Jonckheere 1994b) and a phylogenetic tree was generated (Fig. 2 a). Also, the ORF sequence of NG055 was aligned with the ORF sequences of these *Naegleria* spp. and a phylogenetic tree was generated (Fig. 2b). While *N. minor* does not have a SSUrDNA group I intron, the NG055 intron clusters in both trees with *N. andersoni* and *N. jamiesoni* as in the SSUrDNA tree. This confirms the earlier conclusion that the SSUrDNA intron is of ancestral origin and has evolved with the species, and was lost in different lineages (De Jonckheere 1994b). The intron sequence in the SSUrDNA of strain NG872 will be reported in a paper describing the group I introns in the large subunit ribosomal DNA (LSUrDNA) of NG872 and NG236 as it is quite different (De Jonckheere and Brown, in preparation).

The PCR products from the ITS, including the 5.8S rDNA, are similar in length for all strains except for NG236 (Table 1). In strain NG236 the PCR product is 435 bp long, which is due to an insert in the ITS2. The secondary structure of the ITS2 in NG236 has the same stem-loops (Fig. 3) as the ITS2 in *N. italica*, *N. clarki* and *N. galeacystis*, as reported elsewhere (De Jonckheere, in press), but in NG236 the ITS2 is even longer than in the latter three. The loop of the third conserved stem-loop in the ITS2 of NG236 has the same UUU sequence as found in all other *Naegleria* spp. Due to the large differences in length and sequence of the ITS it is impossible to use alignments for phylogenetic analysis. However, comparison of the ITS sequences of the new thermophilic strains with those of the species to which they are most closely related in SSUrDNA phylogenetic trees provides some additional information. The ITS of strain NG055 is more similar to *N. andersoni* and *N. jamiesoni* than to *N. minor*, which has a very long insert in ITS2. In strain NG872 the ITS1 differs in 11 bp from the *N. lovaniensis* ITS1, while only the third bp in the 5.8SrDNA is different in the two strains. In strain NG236 the ITS2 is longer than in strains NG334 and NG427. Strain NG597 does not have inserts in the ITS2, as is the case in *N. clarki* and *N. italica*, but the ITS1 and 5.8SrDNA are identical in all three strains.

DISCUSSION

The phylogenetic tree inferred from partial SSUrDNA sequences indicates that the genetic distances between NG055, NG236, NG334 and NG427 and the described species are large compared to the distances between the most closely related described species. The percentage

Table 1. Length in bp

Strain	ITS1	5.8S	ITS2	Total length
NG055	34	174	100	308
NG597	33	175	115	323
NG236	34	175	226	435
NG334	35	175	118	328
NG427	46	175	117	338
NG872	36	175	103	314

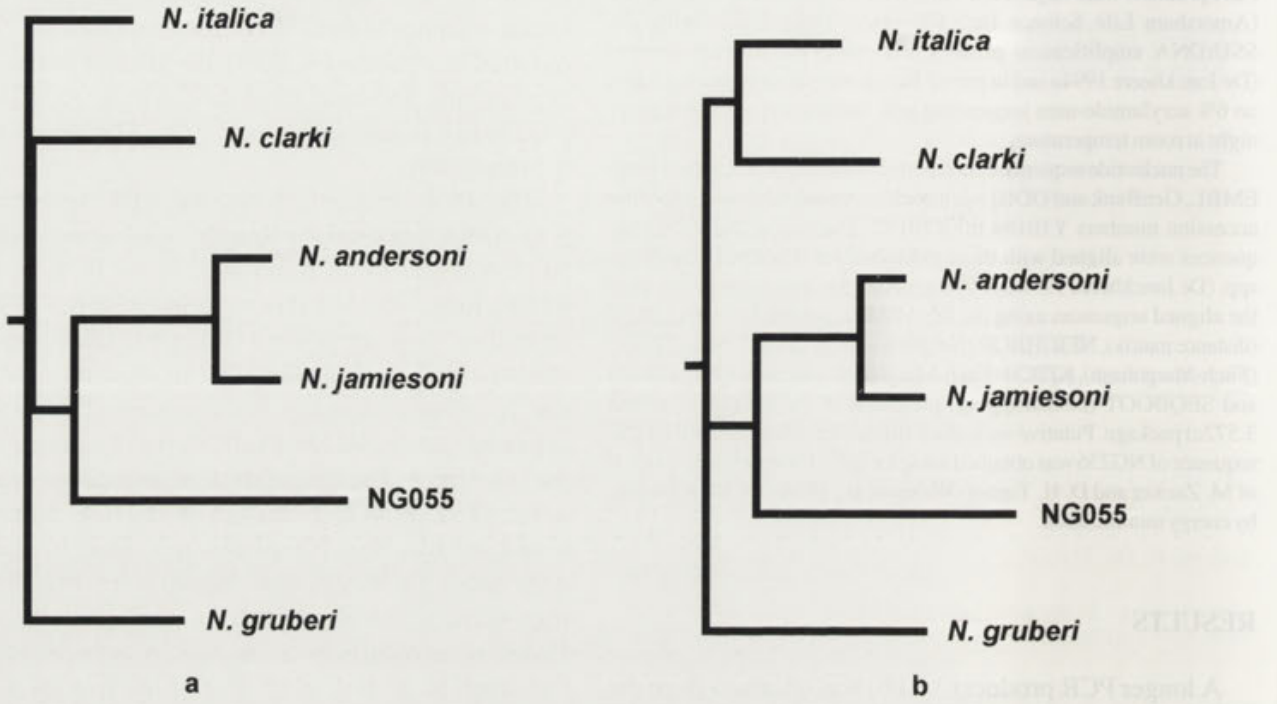

Fig. 2. Phylogenetic trees obtained for the group I DNA sequences (a) and for the amino acid sequences (b) of the ORF in these group I introns

nucleotide differences between these strains and the described *Naegleria* species can be used to infer their separate species status. The sequence of NG055 differs 3.7% from its closest relative *N. minor*, while the difference between *N. andersoni* and *N. jamiesoni* is 4.8% (Table 2). The difference between *N. fowleri* and *N. lovaniensis* is only 1.4% while strains NG236, NG334 and NG427 differ from the former two by 5.8% to 7.0% (Table 3). Therefore, strains NG055, NG236 and NG334 can be regarded as separate species. The difference between NG427 and NG236 is relatively low (0.5%), as is the difference between NG872 and *N. lovaniensis* (0.4 %). However, strain NG872 has a group I intron in the SSUrDNA, while *N. lovaniensis* does not. In addition, uniquely amongst *Naegleria* strains, both NG236 and NG872 have a group I intron in the LSUrDNA (De Jonckheere and Brown, in preparation). When a group I intron is present in the SSUrDNA of a particular species, it was found that all strains of that species have it (De Jonckheere 1993). Therefore, we infer that both NG236 and NG872 represent new species as, in each case, their closest relative does not have group I introns within the rDNA. The recently described *N. pussardi* was considered to be the species most closely related to *N. fowleri* and *N. lovaniensis* (Pernin and De Jonckheere, 1996). However, strains NG236, NG334 and NG427 are much more closely related to the latter. Strain NG872 is even more closely related to *N. lovaniensis* than is *N. fowleri*. Strain NG597 has a 1% difference in SSUrDNA sequence from its closest relatives, *N. clarki* and a

Table 2. Number of nucleotide differences in the partial SSUrDNA (805 positions)

	NG055	*N. minor*	*N. jamiesoni*	*N. andersoni*
NG055	-	30 (3.7%)	63 (7.8%)	55 (6.8%)
N. minor		-	59 (7.3%)	47 (5.8%)
N. jamiesoni			-	39 (4.8%)
N. andersoni				-

Fig. 3. Putative secondary structures for ITS2 of NG055 (a) and NG236 (b) obtained using the mfold program (version 2.3) for RNA folding by energy minimalization

N. gruberi strain NEG-M which was used to obtain the first *Naegleria* SSUrDNA sequence (Accession number M18732) (Table 4). NG597 can, therefore, also be considered a separate species. It also represents the only lineage known in *Naegleria* that contains a SSUrDNA group I intron that has lost the ORF (De Jonckheere and Brown 1994).

The length of the ITS of NG055 is similar to that of most *Naegleria* spp., while in *N. minor*, which is its closest relative based on SSUrDNA sequences, there is a long insert in ITS2 (De Jonckheere, in press). This big difference in ITS sequence confirms the separate species status of NG055. The ITS2 sequence of NG872 is identical to that of *N. lovaniensis*, but the ITS1 differs in 11 bp and there is one different bp in the 5.8SrDNA. This also confirms the conclusion, based on SSUrDNA sequence differences and the presence of an intron in the former, that they can be regarded as separate species. The

Table 3. Number of nucleotide differences in the partial SSUrDNA (805 positions)

	NG236	NG334	NG427	NG872	*N. lovaniensis*	*N. fowleri*
NG236	-	11 (1.4%)	4 (0.5%)	47 (5.8%)	47 (5.8%)	55 (6.8%)
NG334		-	10 (1.2%)	48 (6.0%)	48 (5.9%)	52 (6.5%)
NG427			-	48 (6.0%)	48 (5.9%)	56 (7.0%)
NG872				-	3 (0.4%)	8 (1.0%)
N. lovaniensis					-	11 (1.4%)
N. fowleri						-

Table 4. Number of nucleotide differences in the partial SSUrDNA (805 positions)

	NG579	*N. clarki*	NEG-M	*N. italica*	*N. australiensis*
NG597	-	8 (1%)	8 (1%)	14 (1.7%)	12 (1.5%)
N. clarki		-	8 (1%)	10 (1.2%)	10 (1.2%)
NEG-M			-	11 (1.4%)	11 (1.4%)
N. italica				-	10 (1.2%)
N. australiensis					-

ITS2 of NG236 is much longer than its closest relative NG427, with which it differs by only 0.5% in SSUrDNA sequence, and supports the conclusion they should be treated as separate species. Indeed, it has been observed that strains belonging to the same species have identical ITS sequences, except in *N. fowleri* (De Jonckheere, in press).

It was shown previously that partial SSUrDNA sequences confirm the species status of *Naegleria* spp. which had been described originally using a number of different methods to differentiate them (De Jonckheere 1994a). In this paper we confirm that this partial SSUrDNA sequencing method can replace a plethora of different biochemical methods that were necessary to describe species within a genus that has almost no species-specific morphological markers. Sequencing the ITS can be helpful in deciding on the species status of a strain when the SSUrDNA sequence differences are not considered to be very high (<1.0%). Four new *Naegleria* spp. are described in an accompaning paper (Dobson et al. 1997) based on allozyme, morphometric and physiological characters. Four of the strains studied here were used by Dobson et al., coming to the same conclusion about defining them as new species. Strain NG055 corresponds to *N. carteri*, strain NG236 to *N. morganensis*, strain NG334 to *N. sturti* and strain NG427 to *N. niuginiensis*. Strains NG872 and NG597 will be treated in separate papers by the same authors (Robinson, pers. comm.).

Acknowledgement. We would like to thank Bret Robinson (Australian Centre for Water Quality Research) for providing the strains used in this study.

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Received on 13th June, 1997; accepted on 28th July, 1997

Acta Protozoologica (1997) 36: 279 - 285

ACTA
PROTOZOOLOGICA

On the Morphology and Infraciliature of a New Marine Hypotrichous Ciliate, *Uronychia multicirrus* sp. n. (Ciliophora: Hypotrichida)

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Summary. The morphology and infraciliature of a marine hypotrichous ciliate, *Uronychia multicirrus* sp. n., found in the east China sea near Qingdao (Tsingtao, 36°08'N; 120°43'E) were investigated using protargol impregnation method. Diagnosis of this new species: large *Uronychia* with several macronuclear nodules which are beaded and form a C-shape, size *in vivo* about 120-200 x 80-140 μm; 4 frontal, 5 transverse, 3 left marginal and 3 caudal cirri; base of single "buccal cirrus" long and narrow; 6-8 ventral cirri on right cell margin forming a long cirral row. Constantly 6 dorsal kineties. 11 anterior, 4 posterior adoral membranelles. This organism is closely related to the well-known *U. transfuga*, but differs in having many (instead of 2) ventral cirri, which align in a long row on the right cell margin. Two cells in binary division were observed, which suggests that all the ventral cirri (right marginal ones) derive from the right-most cirral anlage during morphogenesis.

Key words: Hypotrichida, marine ciliates, morphology, new species, *Uronychia*.

INTRODUCTION

The genus *Uronychia* is one of the most well-known ciliates, which is found worldwide in marine and saltwater biotopes and was frequently described (Müller 1786; Stein 1859; Quennerstedt 1867; Wallengren 1900; Buddenbrock 1920; Young 1922; Mansfeld 1923; Wang and Nie 1932; Kirby 1934; Wang 1934; Ozaki and Yagiu 1941; Fenchel 1965; Reiff 1968; Kattar 1970; Agamaliev 1971; Borror 1972a,b; Wilbert and Kahan 1981). In terms of their species separation and terminology, however, these organisms possibly belong to one of the most confused groups ever, even though revision and quite a few studies concerning their taxonomy, morphology and morphogenesis have been conducted rather recently (Curds and Wu 1983, Dragesco and Dragesco-Kernéis 1986, Hill 1990, Valbonesi and Luporini 1990, Petz et al. 1995, Wilbert 1995, Song 1996). This confusion has several causes: i - in many early studies, useful data necessary for describing, such as shape of nuclear apparatus and cell size, were insufficiently given or lacking; ii - many descriptions or revisions are based either on superficial observations or on the second-hand materials which had been generated by other researchers; iii - in previous

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works, attention had hardly been paid to the dissimilarities of the buccal apparatus, which is actually species-specific; iv - *Uronychia*-species possess a rather similar body form, cell size and other consistent features which had been overlooked (or mixed with one another) by many previous authors, and v - all species have very similar biological characteristics (either behaviour or almost every living morphological feature except size).

Up to 1983, about 8 species have been described (Müller 1786, Dujardin 1841, Claparède and Lachmann 1858, Wallengren 1900, Calkins 1902, Pierantoni 1909, Buddenbrock 1920, Young 1922, Taylor 1928, Kahl 1932, Bullington 1940, Fenchel 1965), while in the latest revision by Curds and Wu (1983), only 4 species have been recognized: *U. transfuga, U. setigera, U. binucleata* and *U. magna*. Since then, one more species, *U. antarctica,* was described (Valbonesi and Luporini 1990), which is, however, very likely conspecific with *U. binucleata* (Song and Wilbert, in press).

During the project performed for faunistic studies on marine ciliates in North China, over 30 *Uronychia*-populations belonging to different species have been obtained and studied. As a result, some new conclusions were revealed (Song and Wilbert, in press). These include: 1 - the presence of two "flagella-like" cirri within the buccal field near the cytostome which were mentioned by some previous authors (Calkins 1902, Young 1922, Curds and Wu 1983) and was used as a diagnostic character for some species, is certainly an optical illusion. They are visible only *in vivo*, but in fact, are formed by the rolling/folding of cilia at the margin of the paroral membrane. No corresponding structure was revealed by protargol staining; 2 - the number of macronuclear nodules is pretty stable and is definitely a good character for identification of species as already suggested (Petz et al. 1995); 3 - the spurs (especially the lateral and posterior ones) are variable from inconspicuous to very considerable in all species; 4 - the pattern of the buccal apparatus and the number of basal bodies within the dorsal kineties are highly stable at the species level; 5 - the cell size is also relatively constant, though it may vary within definite ranges, likely affected by limited diet or improper external environment (Song and Wilbert, in press). These details suggest that all known *Uronychia*-species should be assigned to three revised morphospecies: *U. transfuga* (Müller, 1786); *U. setigera* Calkins, 1902 and *U. binucleata* Young, 1922 (Song and Wilbert, in press).

From the samples collected in the same locations, another species with beaded macronucleus-segments and many ventral cirri ("right marginal cirri"), was isolated and investigated. After careful comparison, I believe that it should be considered as a new species, *Uronychia multicirrus*.

MATERIALS AND METHODS

Samples were collected off the coast of Qingdao (Tsingtao, 36°08'N; 120°43'E), China, using polyurethane foam units (PFU). *Uronychia multicirrus* was found (11.21.1993) in a mesosaprobic pond used for storing marine shellfish. The salinity in this pond is about 32 $^{o}/_{oo}$, water temperature 5-13°C and pH is between 8.1-8.3.

Field specimens were isolated and kept in pure culture for the investigations. All species were carefully observed *in vivo* using an oil immersion objective and differential interference contrast microscopy. Protargol silver impregnation (Wilbert 1975) was applied to reveal the infraciliature. Counts and measurements on stained specimens were performed at a magnification of 1250x. Drawings were made with the help of a camera lucida.

Systematics and terminology are mainly according to Corliss (1979) and Kahl (1932).

RESULTS AND DISCUSSION

Uronychia multicirrus sp. n. (Figs. 1-7, Table 1)

Syn. *U. transfuga sensu* Dragesco et Dragesco-Kernéis, 1986

Class: Polyhymenophora Jankowski, 1967

Order: Hypotrichida Stein, 1859

Family: Euplotidae Ehrenberg, 1838

Genus: *Uronychia* Stein, 1859

Diagnosis: large *Uronychia* with several (more than 4) macronuclear nodules which are beaded and form a C-shape, size *in vivo* 120-200 x 80-140 μm; 4 frontal, 5 transverse, 3 left marginal and 3 caudal cirri; 6-8 ventral cirri along right cell margin. 11 anterior, 4 posterior adoral membranelles. Base of "buccal cirrus" long and narrow. Constantly 6 dorsal kineties.

Type location: found on 11. 21. 1993 in a mesosaprobic pond used for storing marine shellfish, in Taipingjiao (the Peace Cape) off Qingdao (Tsingtao, 36°08'N; 120°43'E), P. R. China.

Type specimens: one holotype as a slide of protargol impregnated cells has been deposited in the Laboratory of Protozoology, College of Fisheries, Ocean University of Qingdao, China.

Description: *in vivo* about 120-200 x 80-140 μm, but usually 150-180 μm long, shape more or less elongated

Table 1. Morphometrical data of *Uronychia multicirrus*. All data are based on protargol impregnated specimens. Abbreviations: CV - coefficient of variation in %, Max - maximum, Min - minimum, n - number of cells measured, SD - standard deviation, SE - standard error of mean value. Measurements in µm

Character	Min	Max	Mean	SD	SE	CV	n
Length of body	124	181	148.1	18.20	5.76	5.5	10
Width of body	96	123	108.9	9.08	3.03	8.3	9
Length of buccal field	95	125	110.5	10.76	3.40	9.7	10
Number of macronuclear nodules	5	9	6.5	1.10	0.27	16.9	16
Number of micronuclei	1	1	1	0	0	0	7
Number of membranelles in anterior part of adoral zone	11	11	11	0	0	0	10
Number of membranelles in posterior part of adoral zone	4	4	4	0	0	0	16
Number of frontal cirri	4	4	4	0	0	0	11
Number of transverse cirri	5	5	5	0	0	0	16
Number of ventral cirri	6	8	7.1	0.54	0.16	7.6	11
Number of left marginal cirri	3	3	3	0	0	0	16
Number of caudal cirri	3	3	3	0	0	0	16
Number of dorsal kineties	6	6	6	0	0	0	16

rectangular; right side almost straight while left margin slightly convex (Figs. 1, 3). Dorso-ventrally about 1:2 flattened (Fig. 4). As in other known *Uronychia*-species, 2 concaves in posterior portion of cell when viewed ventrally, where transverse and left marginal cirri insert. On dorsal side a third depression corresponding position of caudal cirri. Spur-like protrusions generally inconspicuous, including 2 anterior, 1 lateral (on left) and 2 posterior (Figs. 2-4). As in other congeners, length of spurs somewhat variable (especially lateral one on left cell margin) and easily overlooked, even when observed at high magnifications.

Cytoplasm greyish to dark grey depending on food conditions, usually comprising many to numerous tiny granules (<1.5 µm). Food vacuoles low in number, containing mainly flagellates or small ciliates (Fig. 1). Contractile vacuole not recognized.

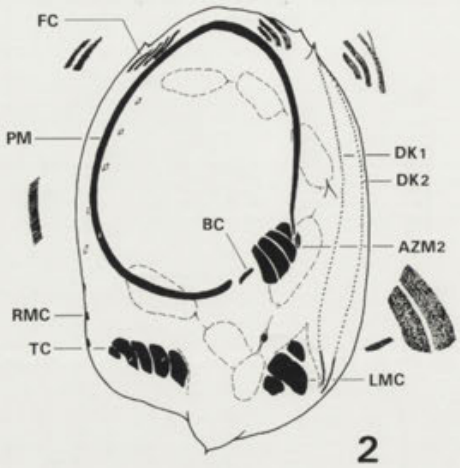
Usually 5-6 macronuclear nodules (Ma), beaded and always connected by funiculus forming C-shape, each nodule ellipsoid to elongate or even sausage-shaped (Figs. 2, 5). One spherical micronucleus (Mi) at lower-left of macronuclei.

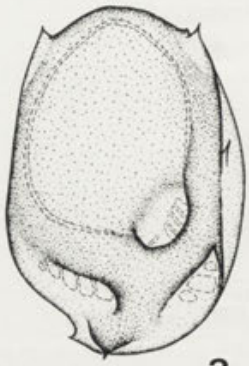
Movement characteristical for *Uronychia*: rapidly jumping sideways or backwards, swimming very fast while rotating around its longitudinal axis. During pause, adoral membranelles and cirri stiffly spread.

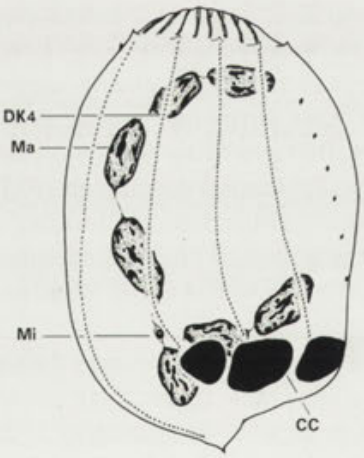
Buccal field enormous, extending over 60-70% of body length. Constantly 11 adoral membranelles in anterior part (AZM_1), mostly extending onto dorsal side (Figs. 2, 5); base of right-most ones (4-5) slightly shorter, each with one branched kinety in sideways (Fig. 2, inset upper right); cilia of membranelles about 20-25 µm long. Posterior part of adoral zone (AZM_2) composed always of 4 long membranelles, in which basal bodies are irregularly aligned in rows (Fig. 2, inset). One cirrus-like membranelle (named buccal cirrus here, BC) apart from AZM_2, its base about 6-8 µm long (Fig. 2). Paroral membrane (PM) mighty, surrounding buccal field and almost forming a closed circle, right end near buccal cirrus, sometimes slightly crook-like (Fig. 2); cilia of PM about 50-60 µm long (Fig. 1).

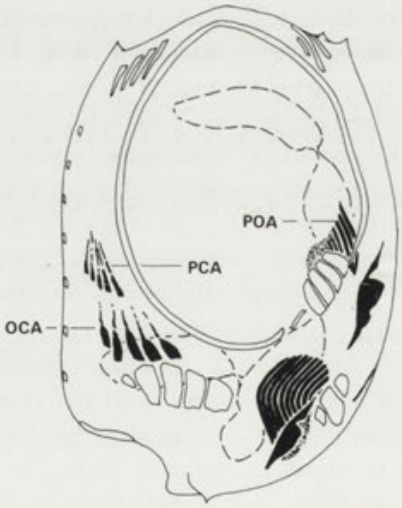
Somatic ciliature typical for genus, constantly 4 long and narrow frontal cirri (FC) resembling short membranelles, bases about 12 µm long, in anterior right area of body. On right border of cell, 6-8 ventral cirri (" right marginal cirri, RMC " , Figs. 2, 5) aligned in one long row which extends anteriorly to dorsal side (q.v. text below and Figs. 6,7). Cilia of both frontal and ventral cirri about 20-30 µm long. Always 5 transverse cirri (TC), 4 left ones considerably enlarged and about 60-90 µm long, right-most cirrus small and inconspicuous (Figs. 1,2). Three prominent left marginal cirri (LMC), about 70-100 µm long, curved at distal end. Three mighty, hook-like caudal cirri (CC) about 70-100 µm long, on right dorsal side (Fig. 5).

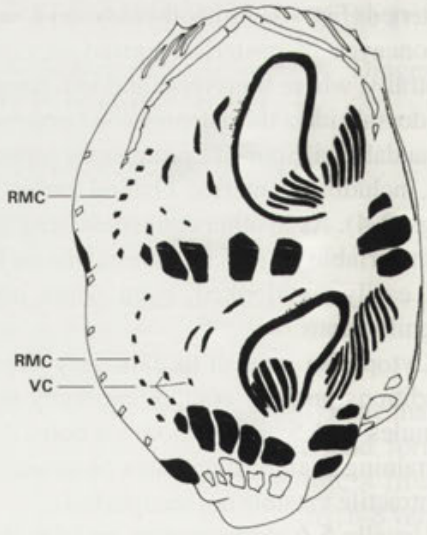
Always 6 dorsal kineties (DK), as in all other known species of this genus, in narrow longitudinal grooves of pellicle, consisting of closely arranged dikinetids. Usually dorsal kinety 1 and 2 on ventral side, both of which terminate posteriorly in a short row consisting of tightly











Figs. 1-5. *Uronychia multicirrus* sp. n. *in vivo* (1,3,4) and after protargol impregnation (2, 5). 1 - ventral view of a typical individual; 2 - infraciliture of ventral side, inset: anterior adoral membranelles (upper-right), note the branched kinetid row; frontal cirri (upper-left); portion of paroral membrane in anterior part of adoral zone (lower-left); buccal cirrus and membranelles in posterior part of adoral zone (lower-right); 3 - ventral view showing the cell surface and spurs; 4 - lateral view; 5 - dorsal view of infraciliature and nuclear apparatus. Abbreviations: AZM_2 - posterior part of adoral zone of membranelles, BC - buccal cirrus, CC - caudal cirri, DK1,2,4 - dorsal kinety 1,2,4, FC - frontal cirri, LMC - left marginal cirri, Ma - macronucleus, Mi - micronucleus, PM - paroral membrane, RMC - right marginal cirri (= ventral cirri), TC - transverse cirri. Scale bar - 100 µm

Figs. 6-7. Ventral views of an early and a middle morphogenetic stage of *Uronychia multicirrus* sp. n., after protargol impregnation. For details see text. Abbreviations: OCA - opisthe's cirral anlagen, PCA - proter's cirral anlagen, POA - proter's oral primordium, RMC - right marginal cirri (= ventral cirri), VC - ventral cirri

Table 2. Morphological comparison of related *Uronychia*-species. Data according to the author

Species	Cell length *in vivo* (in µm)	Number and appearance of Ma segments	Base of buccal cirrus (in µm)	Body shape	No. basal body pairs in left- most dorsal kinety	No. ventral cirri*
Uronychia transfuga (Müller, 1786)	110-250	9-11 beaded, forming a C-shape	10-14 long	oblong and rectangular, lateral spur inconspicuous	*ca* 50	2
Uronychia binucleata Young, 1922	70-120	always 2, elongated sausage-shaped	short, *ca.* 3 long	oval to rectangular, lateral spur inconspicuous	*ca* 30	2
Uronychia setigera Calkins, 1902	40-70	1-2, (mostly 2) short sausage-shaped	short, 2-3 long	oval to short rectangular, usually 1 prominent lateral spur	*ca* 15	2
Uronychia multicirrus sp. n.	120-200	5-9 beaded, forming a C-shape	7-8 long	oblong and rectangular, lateral spur usually inconspicuous	*ca* 50	6-8

* Ventral cirri = right marginal cirri.

spaced basal bodies; kinety 3 extending over entire length of body, others only to caudal cirri (Fig. 5).

Comparison with related species: according to the revised description by Song and Wilbert (in press), *Uronychia magna* Pierantoni, 1909 should be synonymized with *U. transfuga sensu* Song and Wilbert *non sensu* Curds and Wu (1983). *U. transfuga* is thus the only one within the genus, which has both beaded nuclear apparatus (macronuclei) and other morphological features similar to this new species (Stein 1859, Buddenbrock 1920, Young 1922, Kahl 1932, Wilbert and Kahan 1981, Curds and Wu 1983, Wilbert 1995).

Compared with *Uronychia transfuga*, *U. multicirrus* is characterized by possessing a long row of ventral cirri (6-8 vs. constantly 2 cirri). Additionally, the posterior portion of kinety 1 in *U. multicirrus* comprises a row of tightly spaced basal bodies, while in *U. transfuga,* it terminates with a narrow field where basal bodies are scattered forming a disordered patch (Song and Wilbert, in press).

Dragesco and Dragesco-Kernéis (1986) described a form from Bénin, Africa, which they called *Uronychia transfuga*. According to its infraciliature, especially the characteristic long row of ventral cirri and the low number of the macronuclear nodules, it is almost no doubt that their organism should be conspecific with *U. multicirrus* (Table 2).

Discussion: in all known *Uronychia*-species, ventral cirri in non-dividing period are always as 2 small ones on the right cell margin at about the mid point of cell (Kahl 1932, Borror 1972a, Wilbert and Kahan 1981, Curds and Wu 1983, Dragesco and Dragesco-Kernéis 1986, Hill 1990, Petz et al. 1995, Wilbert 1995, Song and Wilbert, in press). During morphogenesis of other congeners, late dividers have often some "surplus" (almost always 3) ventral cirri, which are present only temporarily and will be resorbed soon after completion of division (Hill 1990, Song 1996, Shi and Song, in press). These surplus ventral cirri, which are also present in this population, are always positioned anterior to the transverse ones (Fig. 7, VC).

Based on the specimens shown in Figs. 6-7, the cirri on the right margin are, in terms of their origin, actually ventral cirri which derive from the right-most (the 5th) frontoventral-transverse cirral anlage. So the differentiation of cirri may be as follows:

Cirral anlagen	UM	I	II	III	IV	V
Cirri	1	3	3	2	2	7-9

i.e. 3 frontal cirri develop from anlage I and II respectively (the 4th FC originates from the primordium of the undulating membrane); 3 "temporary" ventral cirri (will be resorbed after division) come from anlage II, III and IV, while 6-8 "permanent" ventral cirri derive from the anlage V and migrate to the right cell margin (RMC in Fig. 7). Each anlage forms one transverse cirrus.

By contrast, in all other known congeners, 5 cirral anlagen generate consistently 13 (instead of 17-19) frontoventral-transverse cirri with the mode of "3, 3, 2, 2, 3" (from anlage I-V) (Wallengren 1900, Hill 1990, Song 1996, Shi and Song, in press), i. e. the anlage V forms only 2 (vs. 6-8 in *U. multicirrus*) "permanent" ventral cirri for the daughter-cells. To evaluate the taxonomic importance of this diversity in - respect to morphology rather than to the morphogenetic pattern - might await further understanding and comparison with other forms (including members of euplotids and other related taxa) since, as we know now, in many higher hypotrichs the number of cirri segregated from a definite anlage is generally very stable (or varies within a pretty limited range) at genus level (e.g. oxytrichiids, *Euplotes, Aspidisca, Diophrys*) but different in some others (e.g. *Gonostomum, Gastrostyla, Discocephalus*) (Borror 1972b, Hemberger 1982, Foissner 1996). *Uronychia*-species, with the exception of *U. multicirrus*, show a highly stable pattern of cirrus formation (Wallengren 1900, Hill 1990, Song 1996, Shi and Song, in press).

Acknowledgements. This work belongs to the project supported by the "Outstanding Youth Foundation of China" (Project number 39425002). I would like to thank Mr. Xiaozhong Hu, graduate student in the College of Fisheries, Ocean University of Qingdao, for collecting material and some technical assistance. I am greatly indebted to Prof. Dr Phyllis C. Bradbury, Department of Zoology, North Carolina State University, USA, for institutional support. My thanks are also due to Ms. Julie M. Kemper, Department of Zoology, North Carolina State University, for kindly reading the English draft.

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Received on 6th February, 1997; accepted on 24th June, 1997

Acta Protozoologica (1997) 36: 287 - 294

ACTA
PROTOZOOLOGICA

Morphology and Biology of a New Species of *Euplotes*, *Euplotes plicatum* sp. n. (Ciliophora: Euplotidae)

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Summary. *Euplotes plicatum* sp. n. was isolated from sandy sediment collected from the harbor of Lyttelton (New Zealand South Island). Its main diagnostic trait is a „pleated" silhouette of the cell dorsal surface generated by 7 (more rarely, 8) prominent and distally bent ridges. Other distinctive traits are: (1) an average size of 50x32 µm; (2) a pattern of 10 frontoventral, 5 transverse, and 4 caudal cirri; (3) 22-25 adoral membranelles; and (4) an argyrome of double type with 10 kineties. A system of multiple mating types, each one specified by one gene expressing serial dominance over its alleles at the apparently single genetic locus *mat*, controls mating interaction of *E. plicatum*.

Key words: ciliate taxonomy, conjugation, *Euplotes* morphology, mating types.

INTRODUCTION

Among ciliates, the genus *Euplotes* has apparently no counterpart as regards variety of species, world-wide distribution, and adaptive plasticity. In these last two decades, over 20 new *Euplotes* morphospecies have been described in addition to the 51 classified by Curds in his guide of 1975 to *Euplotes* taxonomy. A survey of ciliates from Antarctic and circum-Antarctic marine habitats allowed us to identify, at least, four new *Euplotes* species. The description of three of them, *E. focardii*, *E. euryhalinus*, and *E. nobilii*, have been previously reported (Valbonesi and Luporini 1990a, b); here, we describe the morphology and some basic aspects of the biology of the forth one, *E. plicatum* sp. n. This species has already been the subject of a study on the organization and evolution of *Euplotes* argyrome patterns, in which it was indicated as *Euplotes* sp. (Valbonesi and Luporini 1995).

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MATERIALS AND METHODS

Collection and cultures

Specimens of *E. plicatum* were isolated from samples of sandy sediment, collected from the harbor of Lyttelton (Christchurch, New Zealand) by means of a „Petersen" dredge hauled at a depth of 20 m. They were fed with green algae *Dunaliella tertiolecta*, grown in natural sea water (salinity 34-35 ‰; pH 8.2-8.3) enriched with Walne medium and expanded in cultures which are maintained in laboratory at room temperature.

Microscopy

Cells for scanning electron microscopy were fixed for 30 min, at 4°C, using a modified Parducz solution made by mixing 6 parts of 2% OsO_4

(w/v) in sea water (instead of distilled water) and 1 part of saturated aqueous solution of $HgCl_2$. Fixed cells were then (1) washed with 0.1 M Na-cacodylate buffer, (2) mounted on cover-slip fragments previously treated with aqueous solution of 0.1% (w/v) L-polylisine (3) dehydrated in a graded ethanol series, (4) quickly submerged in Freon 113, (5) critical point-dried in an Emscope CPD 750 apparatus, and (6) coated with gold in an Agar Aids sputter coater.

Cells were measured (with the aid of a calibrated ocular micrometer mounted on an optical microscope) after fixation with glutaraldehyde vapor, and analyzed for their cortical structures after silver-staining carried out essentially according to Corliss (1953). Semi-thin sections were prepared from cells first fixed (45 min at 4°C) in 2.5% (v/v) glutaraldehyde and then (30 min at 4°C) in 1% (w/v) OsO_4 in sea water. Fixed cells were then (1) washed 3 times in 0.1 M Na-cacodylate buffer (pH 7.2) with decreasing concentrations of sucrose (12%, 6%, and 3%), (2) concentrated over a film of 2% agar, (3) dehydrated in a graded ethanol series and 100% acetone, (4) embedded in Spurr low viscosity resin, (5) cut with an LKB ultratome, and (6) stained with methylene blue. Cell nuclear apparatus was examined after Feulgen staining.

Assays

Mating type tests were carried out in 3-depression spot slides, using about 500 cells of each strain in 0.5 ml of medium. The intensities of mating reactions were measured, as percentage of cells united in pairs, in samples removed from the mixtures and fixed with Sanfelice fluid. Heterotypic *versus* homotypic mating pairs (i.e., pairs formed between genetically different cells or between identical ones) were distinguished by pre-staining (30 min) with DAPI (concentration, 1 µg/ml) cells of either one of the two cell cultures involved in a mating mixture, and examining (under a fluorescence microscope) samples of pairs after fixation with an ethanol-acetic acid (3:1) solution.

Terminology

Numbers have been applied to cirri and kineties of *E. plicatum* according to Wallegren's system as modified by Gates (1977), with the leftmost (or post-oral) kinety marked as No. 1.

RESULTS AND DISCUSSION

Diagnosis of *Euplotes plicatum* sp. n.

Marine, small-size *Euplotes*. Cell dorsal surface with a pleated profile due to 7 (rarely, 8) prominent ridges sharply bent distally. Ten fronto-ventral, 5 transverse, and 4 caudal cirri. Adoral zone with 22-25 ciliary membranelles. Ten kineties and an argyrome of double type, with uniform rows of polygons. One hook-shaped macronucleus and one spherical micronucleus.

Type specimens

The holotype and a paratype of *E. plicatum* of silver-stained cells have been deposited at the British Museum (Natural History) in London (the accession number has not yet been assigned). A reference strain (NZ_2) has been deposited at the Culture Collection of Algae and Protozoa (the Windermere Laboratory, Ambleside, Cumbria LA22 OLP, UK) and cultures of *E. plicatum* are available from our laboratory.

Etymology

The species denomination is derived from the Latin „*plicatum*" (pleated); it was chosen to emphasize the peculiar pleated silhouette of the cell dorsal surface.

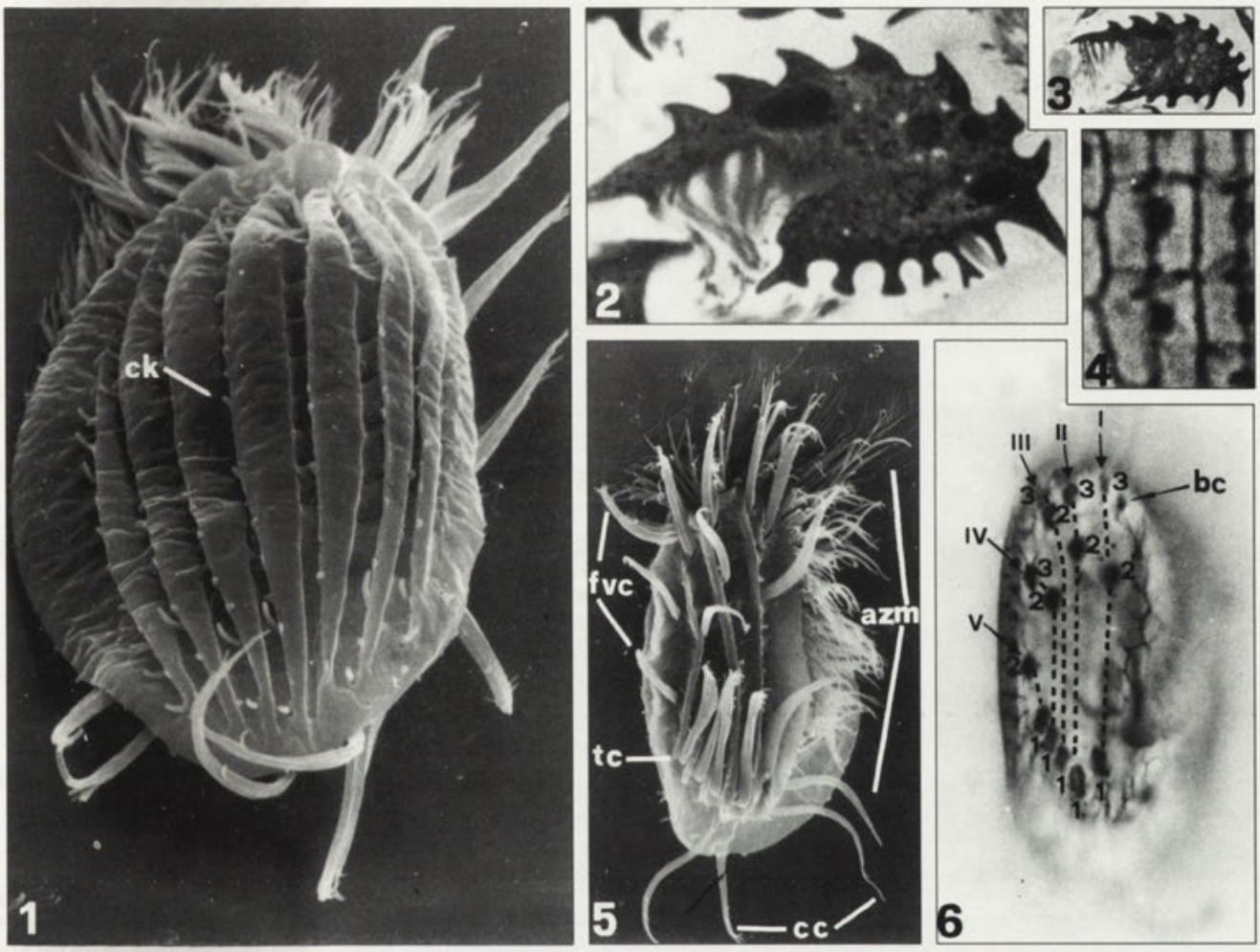
Morphology

The cell body is oval shaped, with the left margin more convex than the right one. The dimensions vary from 42 to 55 µm in length and from 24 to 40 µm in width.

The cell dorsal surface (Fig. 1) has a pleated aspect due to 7 broad and prominent ridges showing their distal portions sharply bent towards the left side of the cell (Fig. 2). One additional ridge, making a total of 8, located on the right half of the cell surface, has been observed in a small percentage of specimens (Fig. 3). Close to left side of each one of these ridges there is one kinety formed by ciliferous kinetosomes, usually amounting to 14 in the central (or 5th) kinety. Three other kineties, one dorsal close to the cell right margin and 2 ventro-lateral, align non-ciliferous kinetosomes. The argyrome (Fig. 4) is of double type, with elongated polygons (that reflect the boundaries of the alveolar sacs underlying the plasma membrane) similar in size and shape. These polygons amount to 15 in the two centralmost interkinetal rows.

The cell ventral surface (Fig. 5) is stretched by 4 extended (or complete) and 2 shorter (or incomplete) ridges. It bears a peristomial field that occupies about two-thirds of the body length and is surrounded by 22-25 adoral membranelles. The first complete ridge (counting from the left side of the body) is the broadest one. It runs adjacent to the inner border of the peristomial field and terminates posteriorly on the left of cirrus 1/I. The second and the third ones separate cirri of streaks I and II, and of streaks II and III, respectively. The fourth one lies between cirri 2 and 3 of streak IV and terminates posteriorly on the right side of cirrus 1/V. The two incomplete ridges are confined to the posterior end of the cell ventral surface, where they emerge at either side of cirrus 1/IV.

The cirral pattern (Figs. 5,6) consists of 10 fronto-ventral, 5 transverse, and 4 caudal cirri, two of which emerge from the rear of the cell, and two are shifted towards the cell left side.



Figs. 1-6. Morphology of *E. plicatum* sp. n. 1 - scanning electron micrograph of the cell dorsal surface showing the cortical ridges and the associated kineties, x1800; 2,3 - light micrographs of semi-thin transverse sections (stained with methylen blue) of two specimens, the former of which bears seven dorsal ridges (i. e., the species-standard number), and the latter, exceptionally eight; x2180 and x800, respectively; 4 - light micrograph of a region of the cell dorsal surface of a silver-stained specimen showing the argyrome of double type with two interkinetal rows of similar polygons, x2160; 5 - scanning electron micrograph of the cell ventral surface carrying cirri, cortical ridges, and adoral membranelles, x1040; 6 - light micrograph of the cell ventral surface of a silver-stained specimen on which Arabic and Roman numbers and dashed lines have been superimposed, according to Wallengren's system as modified by Gates (1977), to illustrate the spatial disposition of cirri and their developmental connections, x1150. Abbreviations: azm - adoral zone of ciliary membranelles; bc - buccal cirrus; ck - central kinety; fvc, tc, and cc - frontoventral, transverse, and caudal cirri, respectively

In starved cells, arrested in the G_1 (or, G_0) stage of the cell cycle, the nuclear apparatus consists of one hook-shaped macronucleus and one spherical micronucleus lying close to the left anterior edge of the cell body.

Taxonomic identification

Euplotes plicatum falls into a group of 27 *Euplotes* species characterized by 10 fronto-ventral cirri and a dorsal argyrome of double type. (We refrain here from introducing a further distinction of these species into the double-*patella* and double-*eurystomus* subgroups, since this distinction is taxonomically unreliable, as earlier discussed, in particular by Gates and Curds 1979, and Valbonesi and Luporini 1995).

The diagnosis of these species has been essentially based on the comparative analysis of the morphological traits listed in Table 1. These traits are reliable only if they diverge sufficiently from one to another species to warrant their use, and are considered as a whole (not individually). Most of them are in fact subjected to marked variations even among individuals of the same species (Curds 1975, Hill and Reilly 1976, Gates 1978). Thus, measures and qualitative attributes of each one of the 27 *Euplotes* species of the same group as *E. plicatum* have been first

Table 1. Diagnostic traits of *E. plicatum* and 27 *Euplotes* species characterized by 10 frontoventral cirri and an argyrome of double type

Species	Body length and width (μm)	Body shape	Kineties (*n*)	Kinetosomes on the mid-dorsal kinety (*n*)	Adoral membranelles (*n*)	Macronuclear shape	Authors
E. plicatum	42-55 x 24-40	oval	10	14	22-25	hook	original
E. charon #	71-86 x 47-63	ovoid	12	16	65-70	hook	Ehrenberg, 1830
E. harpa *	150-160 x ND	oval	13	40-45	65-70	C or 3	Stein, 1859
E. alatus	40 x 30	oval	8	10-12	26	C	Kahl, 1932
E. balteatus *	30-150 x ND	ovoid	8	11	25-80	C or h-s	Kahl, 1932
E. trisulcatus	40 x 30	oval and narrower at its posterior end	7	11	25-36	C	Kahl, 1932
E. crenosus	50-70 x ND	oval, with an anterior notch	8	23	25-30	C	Tuffrau, 1960
E. inkystans	70-80 x ND	oval, with an anterior notch	10	25	40	C	Chatton in Tuffrau, 1960
E. neapolitanus	130 x 70	ellipsoidal and wider at its anterior end	11	18	65	C	Wichterman, 1964
E. tuffrai	113 x 73	pyriform	9-10	8-11	40-45	C	Berger, 1965
E. octocirratus	55-60 x ND	oval and narrower at its posterior end	7	14	30	C	Agamaliev, 1967
E. antarticus	85-90 x 30-35	elongate	8	ND	30	J	Fenchel & Lee, 1972
E. magnicirratus	54 x 40	ovoid	8	13-17	50	h-s	Carter, 1972
E. polycarinatus	90 x 80	triangular	20-21	23	60-76	3	Carter, 1972
E. rariseta	30-45 x 20-31	ovoid	6	6	23	S	Curds et al., 1974
E. palustris	45-55 x 35-45	oval	8	11-14	25-35	C	Ten Hagen, 1980
E. mediterraneus	63 x 45	ovoid	8	ND (paired)	47-50	h-s	Fernandez-Leborans & Castro De Zaldumbide, 1985
E. platystoma	90-118 x 56-80	ovoid	11	18-21	44-68	h-s	Dragesco & Dragesco-Kérneis, 1986
E.kurekchayi	80-85 x 50-55	oval	8	13-15	40-45	h-s	Aliev, 1987

Table 1. (con.)

E. ogusi	50-55 x 30-32	oval	7	13-15	35-40	3	Aliev, 1987
E. corsica	29-42 x 22-32	oval	7-8	6-9	20-25	C	Berger & Foissner, 1989
E. algivora	40-59 x 24-40	oval, with projection on the right front	6	7-12	28-37	C	Agatha *et al.*, 1990
E. focardii	38-110 x 30-92	ellipsoidal or round	10-12	13-22	45-65	h-s	Valbonesi & Luporini, 1990a
E. euryhalinus	50-62 x 26-38	oval	11	16-18	26-28	h-s	Valbonesi & Luporini, 1990b
E. nobilii	29-43 x 18-29	spindle-shaped	8	9	18-22	C	Valbonesi & Luporini, 1990b
E. acanthodus	90 x 60	oval or inverted triangular with spur on the anterior right	12	17	66	C	Petz, Song & Wilbert, 1995
E. margherensis	50 x 40	oval, with projection on the right front	6	9-11	35-45	hook or 3	Coppellotti & Cisotto, 1996
E. shanghaiensis	75-108 x 55-76	D-shaped or inverted triangular	12-13	21-26	53-58	C	Song, Hill & Warren, (in press)

Data from Valbonesi *et al.* (1987).
* Data from Curds (1975).
ND, not determined.
h-s, horseshoe-shaped

coded into a few states with a wide interval of variability (Table 2), and then jointly analyzed by means of an „unweighted pair-group method using arithmetic averages" (UPGMA) which has been applied to a similarity matrix derived from Gower's similarity coefficients.

This cluster analysis, in spite of its tendency to compress rather than to widen inter-species differences (as a matter of fact, *E. alatus*, *E. algivora*, *E. corsica*, *E. nobilii*, and *E. trisulcatus*, would appear to be not separate from one another), produces a dendrogram showing that *E. plicatum* clearly diverges from all the other species (Fig. 7). The species closest to *E. plicatum* appears to be *E. euryhalinus*, linked at 83% of similarity. This extent of similarity would have been even lower, had *E. plicatum* and *E. euryhalinus* been compared also for the aspect of their dorsal surfaces, nearly smooth in the former versus pleated in the latter. Notwithstanding a recognized value in *Euplotes* taxonomy (Borror 1986, Foissner et al. 1991, Valbonesi and Luporini 1995), a description of the architecture of the cell dorsal surface is not available for every species; thus, this trait could not be included in the cluster analysis.

Mating

Samples from every one of the 10 wild strains collected as representative of *E. plicatum* were mixed in all possible pairwise combinations and these mixtures were then monitored for the appearance of mating pairs. Two strains, NZ_2 and NZ_7, showed higher reciprocal mating compatibility than others (at least 60% of their cells underwent pairing within 2-3 h of mixing) and formed only (or mostly) heterotypic pairs, as detected by pre-labelling samples of either one of them with DAPI before mixing. Thus these strains were chosen to represent two different Mt's (NZ_2 = Mt-I, NZ_7 = Mt-II) and used to study the Mendelian genetics of Mt inheritance of *E. plicatum*.

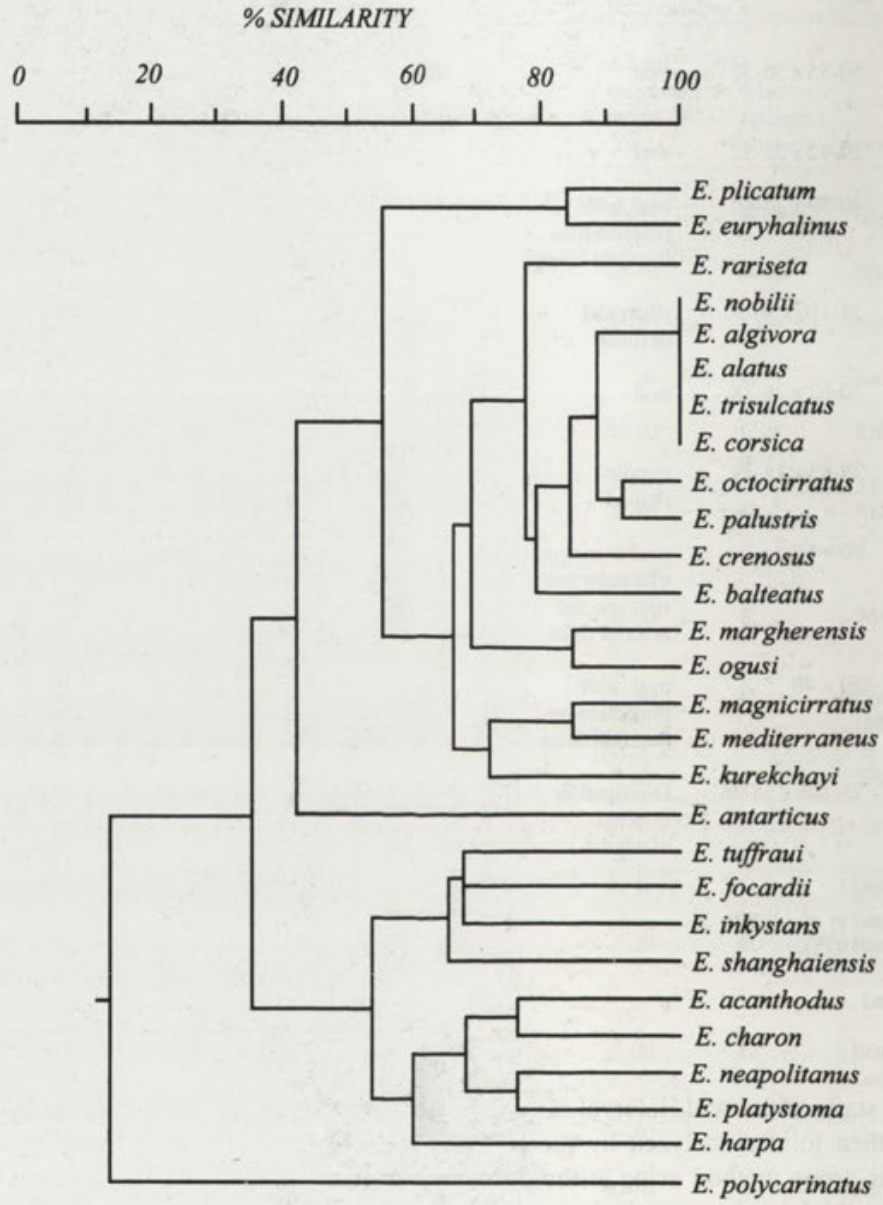


Fig. 7. UPGMA clustering of *E. plicatum* with related *Euplotes* species based on the diagnostic traits listed in Table 1 and coded as reported in Table 2

Eighty-eight F_1 clones were yielded from 100 one-day-old exconjugants singly isolated from a mixture between Mt's I and II, and tested against their own parental strains at maturity, that was manifest 50-60 cell divisions (equivalent to about 2 months) from their postconjugal reorganization. As reported in Table 3, 38 clones were assigned to Mt-I (mating did not occur with the Mt-I parent but did occur with the Mt-II parent), 27 to Mt-II, and 23 (capable of mating with both parents and incapable of mating with one another) to a new (nonparental) Mt (denoted Mt-III).

Based on the notion that Mt's in *Euplotes* are inherited through multiple alleles at the same locus *mat*, where they may be expressed either with relationships of codominance according to the *E. patella* model (Kimball 1942), or serial dominance according to the *E. vannus-crassus-minuta* model (Heckmann 1963, 1964; Nobili 1966), this Mt segregation (fitting with a 2:1:1 ratio between Mt's I, II, and III) was explained by assuming the involvement of at least three *mat* alleles with serial dominance, i. e., *mat*-1>*mat*-2>*mat*-3. To verify this assumption and seek

Table 2. States and coding of the morphological traits of *Euplotes* species listed in Table 1

Traits	States	Coding +
Body length (μm)	30 - 70	S
	71 - 100	M
	101 - 160	L
Body shape*	Triangular	T
	Elongated	E
	Oval	O
	Round	R
Kineties (n)	6 - 8	F
	9 - 13	N
	14 - 23	M
Kinetosomes in the mid-dorsal kinety (n)	6 - 13	F
	14 - 18	N
	19 - 25	M
	26 - 45	E
Adoral membranelles (*n*)	18 - 37	F
	38 - 64	N
	65 - 80	M
Macronuclear shape #	C	C
	3	T
	hook	H

* Triangular: anterior end twice wider than the posterior one. Elongate: body nearly three times longer than wide. Oval (covering also terms such as elliptical, spindle-shaped, pyriform, ovoid, and D-shaped): body nearly twice longer than wide. Round: body as long as wide.
# C-shaped covers also terms such as hoop- and horseshoe-shaped. Hook covers also terms such as J- and S-shaped.
+ Traits are included in the data matrix (used for calculating the Gower's coefficients) with two codes: identical (e.g., S S) or different (e.g., S M) according to whether they fall into one or two states, respectively

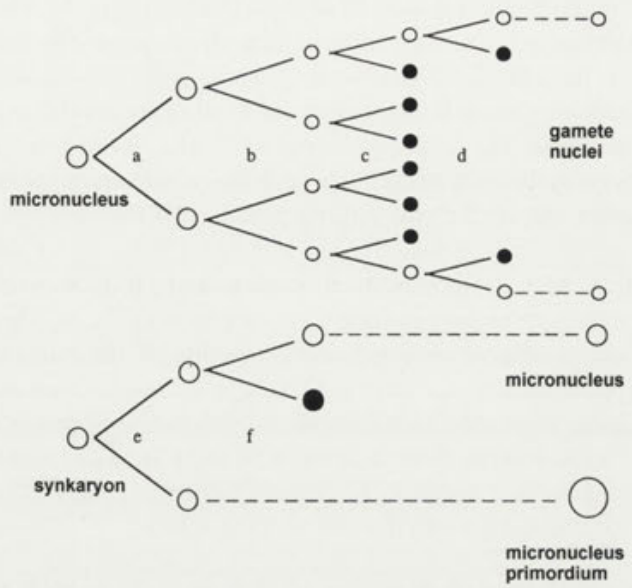


Fig. 8. Diagrams of the nuclear processes related with conjugation of *E. plicatum*. a-d - Preliminary mitosis and pregamic divisions of the micronucleus; e-f - metagamic divisions. White and black circles stand for surviving and degenerating division products, respectively

the *mat* combinations carried by Mt's I, II, and III, one F_1 clone of Mt-III (denoted 1aF_1 in Table 3) was back-crossed to both parental strains and the two sets of offspring clones analyzed for the Mt segregations. In both cases, half clones expressed Mt-III and half either one of the two parental Mt's. Thus, it was concluded that *mat*-1/*mat*-3, *mat*-2/*mat*-3, and *mat*-3/*mat*-3 are the putative *mat* combinations of Mt's I, II, and III, respectively, and that Mt's of *E. plicatum* are inherited according to the *E. vannus-crassus-minuta* model.

Nuclear processes

Cytological analysis of the nuclear processes relative to conjugation showed that *E. plicatum* conforms with most *Euplotes* species with regard to the pre-zygotic divisions of the micronucleus (Raikov 1972). A preliminary mitosis precedes the three pregamic divisions, resulting in the formation of the two gamete-nuclei, each one

Table 3. Mating type segregation in crosses of *E. plicatum*

Crosses	Viability (%)	Tested clones (n)	Mating types of progeny clones			Expected ratio and probability (P) by $\chi 2$
			Mt-I	Mt-II	Mt-III	
NZ_2 (Mt-I) x NZ_7 (Mt-II)	88	88	38	27	23	2 : 1 : 1 P > 0.3
NZ_2 (Mt-I) x 1aF_1 (Mt-III)	84	32	14	0	18	1 : 1 P > 0.3
NZ_7 (Mt-II) x 1aF_1 (Mt-III)	67	25	0	9	16	1 : 1 P > 0.1

from a different quartet of meiotic products (Fig. 8). The synkaryon formation and the first post-zygotic (or, metagamic) division take place in cells that are still united in pairs and have nearly resorbed all the breakdown pieces of the „old" macronucleus. The second post-zygotic division occurs late in 1-day-old exconjugants, when one of the two mitotic products of the first synkaryon division has already started swallowing in the primordium of the new macronucleus, and it originates the new micronucleus and one abortive product (Fig. 8). This pattern of post-conjugal reorganization of the nuclear apparatus of *E. plicatum* appears to be somewhat unusual among most species of *Euplotes*, in which it is one product of the second synkaryon division (Raikov 1972), or of the third one as it occurs in *E. charon* (Valbonesi et al. 1987), that transforms into the new macronucleus.

Acknowledgements. Work financially supported by Italian P.N.R.A. (National Programme of Researches in Antarctica). Thanks are due to Dr. Andrea Brusaferro (University of Camerino) for his valuable help in performing the cluster analysis.

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Received on 7th August, 1997; accepted on 12th September, 1997

Acta Protozoologica (1997) 36: 295 - 304

ACTA
PROTOZOOLOGICA

Intexta acarivora gen. et sp. n. (Microspora: Chytridiopsidae)- Ultrastructural Study and Description of a New Microsporidian Parasite of the Forage Mite *Tyrophagus putrescentiae* (Acari: Acaridae)

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Summary. The microsporidium *Intexta acarivora* gen. et sp. n., a parasite of the gut epithelium of the forage mite *Tyrophagus putrescentiae* (Schrank, 1781) is described based on ultrastructural characteristics. Reproduction is restricted to the production of sporoblasts. The sporont has isolated nuclei, and the external part of the sporont (plasma membrane and peripheral zone of cytoplasm) remains as an envelope collecting the uninucleate sporoblasts. Sporogony is by vacuolation, and mature spores are spherical to slightly ovoid and lack a polaroplast. Microspores (1.3-1.7 µm wide) are dominant, and only a small percentage of macrospores (1.5-2.3 µm wide) were seen. The exospore is composed as a thin (*ca* 16 nm) uniform, electron-dense layer; the endospore is well developed. The polar filament is completely coiled and anisofilar, and in microspores arranged in 2-3 coils (anterior 1-2 wider); macrospores have up to 9 coils (anterior 3-4 wider). The external zone of the polar filament contains tubular material, and the polar sac is without an anchoring disc. The species is compared to previously described microsporidia of mites, and the genus and family affiliations are discussed.

Key words: Acari, *Intexta acarivora* gen. et sp. n., Microspora, taxonomy, *Tyrophagus putrescentiae*, ultrastructure.

INTRODUCTION

The class Arachnida contains more than 60 000 species, most of them terrestrial mites. Many are common and widespread, and several are of economic importance. It might be expected that numerous parasites would have been recorded from this group, but this is not the case. Microsporidia have only been occasionally observed in arachnids, and only from three of the 11 extant orders. Two orders, Araneae (spiders) and Opiliones (harvestmen), host one microsporidian species each (Silhavy 1960, Codreanu-Balčescu et al. 1981) while the remaining 21 named and three unnamed species are parasites of Acari (mites).

All described microsporidia associated with arachnids are species expressing the normal microsporidian cytology (including a long, coiled polar filament and a voluminous polaroplast) - the construction typical of what is normally interpreted to be the most highly evolved microsporidia. The genera *Cryptosporina*, *Gurleya*, *Napamichum* and *Thelohania* each have one known species whereas the remaining species have been assembled either in *Nosema*, *Pleistophora* or the unclassified genus *Microsporidium*. With few exceptions, the descriptions are extremely superficial. Our present knowledge of the genus *Pleistophora* tells us that the mite parasites assigned to this genus obviously do not belong there, for all species so far proven

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to belong to *Pleistophora* are parasites of fish, amphibia or reptiles. It can also be expected that many of the other microsporidia of mites have not found their proper genus position.

Recently, a previously unknown microsporidian parasite was observed in the gut epithelium of the forage mite *Tyrophagus putrescentiae* (Schrank, 1781) obtained from a culture in the Netherlands. The mite is used as a prey species in commercial rearing of the phytoseiid mite *Neoseiulus cucumeris* (Oudemans, 1930). The microsporidium produces small spherical spores, expressing a cytology that differs distinctly from that seen in other microsporidia of mites. The microsporidium, which is new to science, is described briefly herein and its identification and classification are discussed. As no established genus can accommodate the species, a new genus has been erected.

MATERIALS AND METHODS

A mixed culture of *Tyrophagus putrescentiae* and *Neoseiulus cucumeris* were obtained on three occasions in 1992 from a commercial insectary in the Netherlands. Approximately 25 adult females of each species were removed from the shipping containers on arrival and prepared for transmission electron microscopy.

Whole mites were fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 24 to 48 h. Fixative was replaced with 0.12 M cacodylate buffer (pH 7.2) with three changes in 60 min, and tissue was postfixed for 2 h in 1% osmium tetroxide in 0.12 M cacodylate buffer.

Fixed mites were placed in distilled water for 10 min and dehydrated in an ascending ethanol series, followed by propylene oxide: low viscosity Spurr resin (16 h), and low viscosity Spurr resin (24 h). Each solution was changed three times. Individual mites were placed in Spurr resin with a flat mold and cured for a minimum of 16 h in a 60°C oven. Ultrathin sections were cut with a diamond knife and stained using uranyl acetate and led citrate (Reynolds 1963).

RESULTS

Prevalence and pathogenicity

The infection caused no externally visible signs. Three of six randomly selected mites embedded in blocks were infected. Round spores, possibly of this species, were observed in Giemsa-stained smears from batches of *T. putrescentiae* from the Netherlands, and from batches from Canada. None were observed in *N. cucumeris*. The microsporidium was only found in the gut epithelium. Immature stages and mature spores occurred together in the same host cell (Figs. 1-3). The gut epithelium was fairly thin and sometimes heavily loaded with microsporidia (Fig. 2), and microsporidia-filled cells were shed into the gut lumen. Infected cells and host nuclei were not hypertrophic, and no particular associations with host nuclei and mitochondria were observed (Fig. 3). Parasitophorus vacuoles, encapsulation or other defence reactions were not seen.

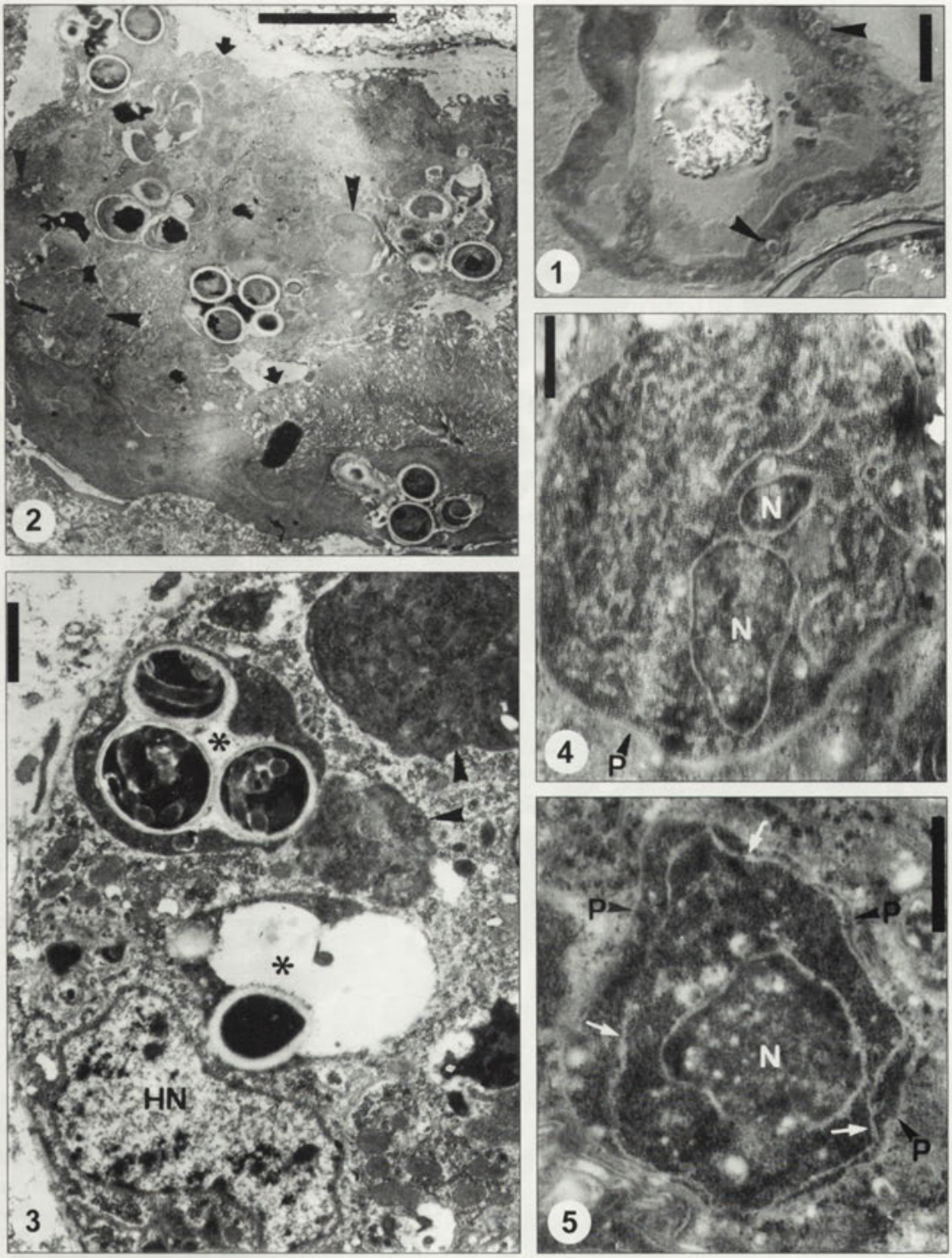
Two other types of protozoa occurred together with the microsporidium in the mites studied. Eugregarines were regularly present in the gut lumen, as well as various developmental stages of another apicomplexan in the fat tissue. Sexual reproduction was not observed and it is uncertain whether it was a neogregarine or an adeleine coccidian. The oocysts contained numerous sporocysts. Consequently it can be excluded that this apicomplexan is related with the *Isospora*-like coccidian of haemogamasid mites described by Mohamed et al. (1987), which was tentatively identified to belong to the life cycle of *Elleipsisoma thomsoni*, a blood parasite of moles.

Presporal stages and life cycle

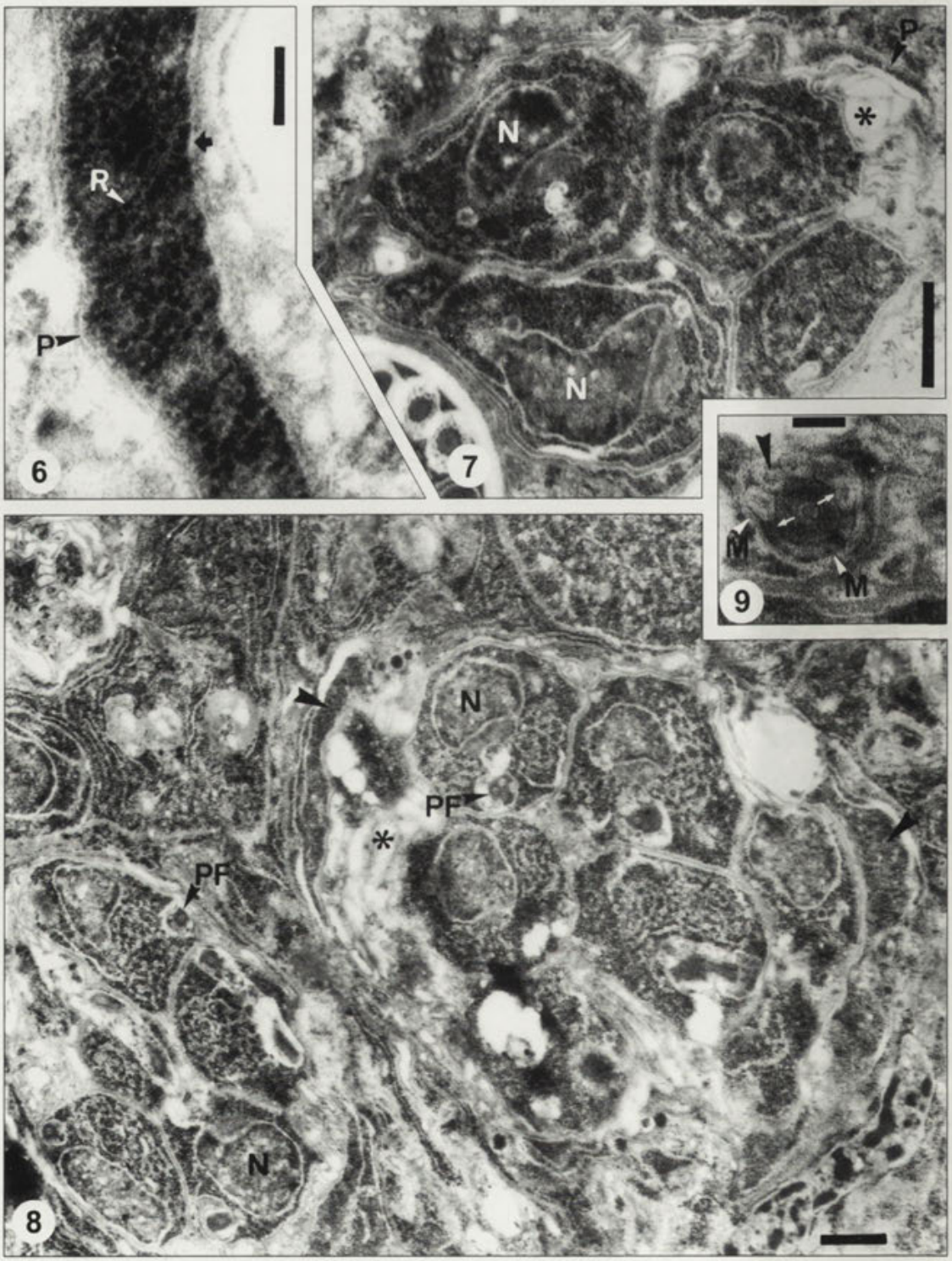
The minute size of the microsporidium made light microscopic observations and measurements rather difficult. The life cycle and cytology of the developmental stages were deduced from the study of ultrathin sections.

All life cycle stages had isolated nuclei. Presporogonic development was not observed, and it is thought that sporogony may be the only reproductive phase. The earliest stages observed were sporonts; plasmodia with a small number of nuclei (Figs. 4, 5). The plasma membrane was *ca* 8 nm thick, consisting as a unit membrane, and lacking external reinforcements. The cytoplasm was fairly electron-dense containing numerous free ribosomes. Nuclei were also fairly electron-dense, and delimited by a nuclear envelope of normal construction: two unit membranes separated by a perinuclear space. In early stages, when only one or a few nuclei were visible in each sectioned sporont, membranes appeared in the cytoplasm (Fig. 4). They grew to a continuous membrane sheath near the periphery of the cell (Fig. 5). By this arrangement, a superficial layer of cytoplasm was enclosed between the plasma membrane and the newly formed internal membrane (Fig. 6) forming a persistent sac in which the spores were produced and collected.

Spores were produced endogenously. In the centre of the cytoplasm vacuoles appeared, separating cell fragments so that each contained one nucleus and a peripheral zone of cytoplasm. Each fragment developed into a sporoblast, and the vacuole membrane was incorporated



Figs. 1-5. Pathogenicity and early development of *Intexta acarivora* gen. et sp. n. 1 - semithin section of the midgut epithlium of *Tyrophagus putrescentiae*; arrowheads indicate spores (methylene and toluidine blue). 2 - ultrathin section of the midgut epithelium; spores are captured inside the cell wall of the sporont, presporal stages are free in the cytoplasm (arrowheads); arrows indicate the brush border. 3 - midgut epithelium cell with sporulated sporonts, enclosing spores, and presporal stages (arrowheads); * indicates the central cavity of the sporont where the spores mature; no particular connection between host nucleus and the parasites. 4,5 - young sporonts, arrows indicate developing internal membranes. HN - host nucleus, N - nucleus, P - plasma membrane. Bars: Fig. 1 - 10 μm, 2 - 5 μm, 3 - 1 μm, 4,5 - 0.5 μm



Figs. 6-9. Early sporogony. 6 - peripheral part of a sporont, cytoplasm rich in ribosomes; arrow indicates the internal vacuole membrane. 7 - sporont with four fairly dense sporoblasts visible in the central cavity, * indicates vacuole. 8 - sporonts (arrowheads point at the dense peripheral zone, * indicates vacuoles) with older sporoblasts, first signs of polar filament visible. 9 - detail of the developing polar filament of a sporoblast, arrows indicate the central part, arrowhead the peripheral tubules; the tubules are formed inside the unit membrane cover. M - membrane, N - nucleus, P - plasma membrane, PF - polar filament, R - ribosomes, Bars: Figs. 6, 9 - 100 nm, 7,8 - 0.5 μm

as the plasma membrane (Figs. 7, 8). The sporoblasts matured into spores without further division. The density of the cytoplasm was reduced during the maturation process, and older sporoblasts appeared more granular than newly formed ones (Figs. 7, 8). The number of spores per sporont was small and irregular. No more than 6 spores were seen in a sectioned sporont.

Mature spores

Two size classes of spores with overlapping ranges were observed. The dominant spore type in sections were microspores (Fig. 10), measuring 1.32-1.78 μm in diameter. Their spore wall was 101-136 nm thick. Macrospores (Fig. 11) were of similar construction, differing only in numerical characters. They measured 1.49-2.33 μm in diameter, and their spore wall was up to 145 nm thick. Macrospores were apparently formed by anomalous sporogony, and always occurred together with microspores (Fig. 11).

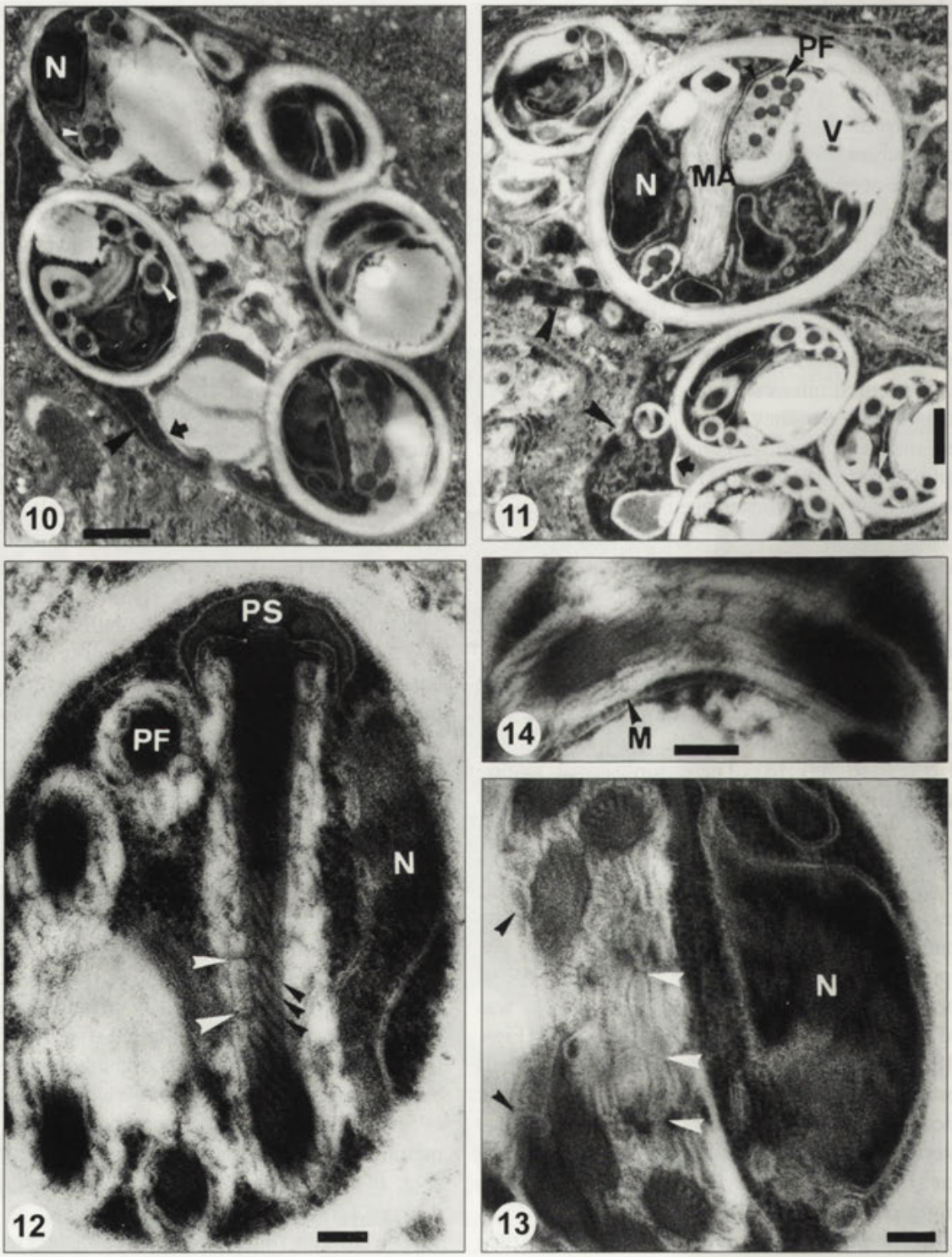
Both spore types were spherical to slightly ovoid (Figs. 1, 10, 11). The nucleus was localized to one side of the spore (Figs. 12, 13), irregular in shape, and in most sections fairly flat or almost hemi-spherical (Figs. 10 - 13). The nuclei of the microspores measured up to 1.1 μm in diameter, whereas nuclei of macrospores up to 1.2 μm. The nucleoplasm was denser at the periphery of the nucleus (Figs. 10, 11, 13). The cytoplasm of mature spores was fairly electron-dense, packed with free ribosomes (Figs. 12, 13); polyribosomes were not seen. The pole opposite the nucleus had a wide, irregular, lucent vacuole with a unit membrane lining. Vacuoles of microspores were up to 1 μm wide, somewhat larger than those found in macrospores.

The polar filament differed from the normal type of microsporidian polar filament both in the way it was arranged in the spore and in constructional details (Figs. 12, 17, 18). It was not divided into an anterior straight part and a posterior tilted section; rather, the complete filament was coiled (Fig. 12). The anterior coils were wider than the posterior ones, and the filament classification is anisofilar (Figs. 10, 11). The filaments of microspores were arranged in 2-3 coils (1-2 wide, 1-2 narrow) (Figs. 10-12). Macrospores had a greater number of coils than microspores up to 9 (3-4 wide, 4-5 narrow). The coils occupied the space between the nucleus and the vacuole.

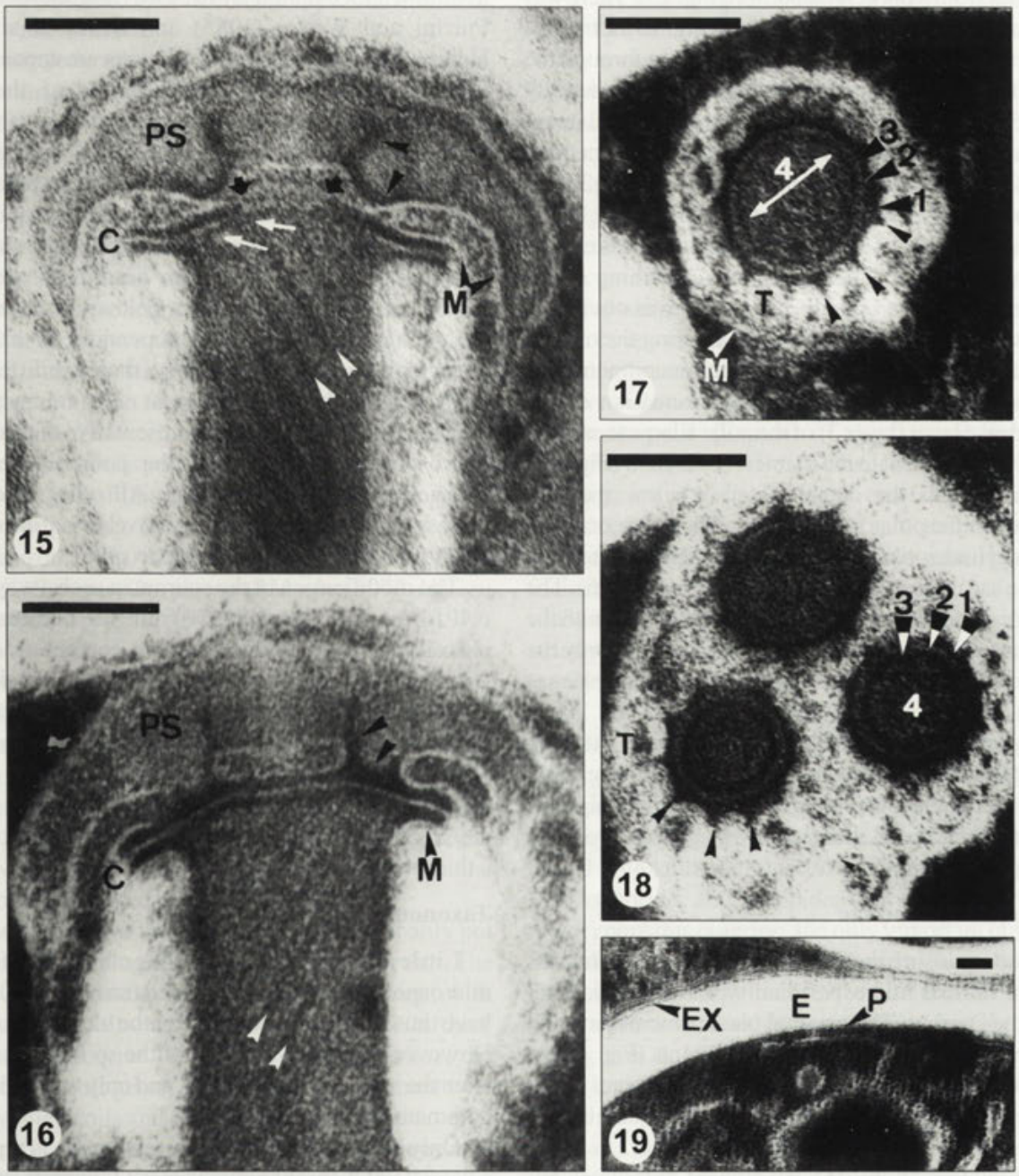
The polar filament had an electron-dense core, comparable to a normal microsporidian filament, and a lucent construction at the surface (Figs. 17, 18). The total width, including the lucent periphery, was 170-238 nm in narrow, 263-272 nm in wide coils of microspores. In both spore types the core of the anterior wide coils measured 136-221 nm, while the core of the posterior coils was 102-128 nm wide. In macrospores, filament coils were often grouped together, coupled, or in greater numbers, covered by the unit membrane sheath (Fig. 11). The core was composed of material of two electron densities; material of the highest density formed two layers about 6 nm wide; one at the surface of the core and one internal (Figs. 17, 18, layers 1 and 3). Between these layers was a zone of moderately dense material (layer 2), about 9 nm wide. The same material filled the centre, where traces of layering could be seen (Figs. 17, 18, layer 4).

In the transversely sectioned filament the lucent surface structures appeared as transversely sectioned tubules of lucent material about 6 nm thick (Figs. 13, 17). In microspores the number was about 12. In macrospores, where coils were grouped, the number of tubules belonging to the individual coils were difficult to count, but appeared to be the same. The dense material at the surface of the core (layer 1) extended into the space between the tubules and gave the transversely sectioned core a star-like appearance (Figs. 17, 18). The tubules followed the core and were always sectioned more or less identically to the core, i.e. transversely sectioned in transverse sections of the filament, longitudinally in longitudinal sections (Figs. 12, 13, 17). The tubules wound around the core. In longitudinal sections, taken at the surface of the core, the dense material between the tubules appeared as an oblique striation with the strands separated by a distance of about 20 nm. The strands were arranged in parallel and at an angle of *ca* 30° to the long axis of the filament (Figs. 15, 16). Longitudinal sections through the tubular layer showed 130-170 nm long tubule sections, which indicated that the tubules were intertwined (Figs. 12, 13). The lucent zone was always distinct, but it was difficult to see how the surface of the translucent external zone was constructed (Figs. 10-12). In sporoblasts, the tubules were initiated below a unit membrane cover about 8 nm thick (Fig. 9). In favourable sections of mature spores, a continuous unit membrane sheath could be seen outside the tubules (Fig. 14). A polaroplast of normal microsporidian type was not formed.

The anterior filament coil was attached to a polar sac filled with moderately electron-dense material (Fig. 12). The widest sectioned polar sac measured 477 nm in diameter. It was externally covered by a unit membrane about 8 nm thick, below which was an electron-dense zone up to 13 nm thick. In longitudinally sectioned coils the externally directed surface of the polar sac was almost crescent-shaped, while the internal surface bent at almost



Figs. 10-14. Mature spores. 10,11 - sporonts with micro- and macrospores, nucleoplasm denser at the periphery; black arrowheads indicate the peripheral zone of cytoplasm, arrows vacuoles. Polar filaments of microspores individually coiled, coils in macrospores grouped under a unit membrane cover, white arrowheads indicate wide coils of the anisofilar polar filament. 12 - microspore, anterior polar filament coil sectioned longitudinally at the periphery of the filament core; white arrowheads indicate sections of tubules, black arrowheads oblique strands of electron-dense material belonging to the external part of the filament core (layer 1). 13 - sections through the tubular layer of the polar filament; black arrowheads indicate transversely sectioned tubules, white arrowheads point at sections of winding tubules. 14 - longitudinal section of a coil; covering membrane visible. M - membrane, MA - macrospore, N - nucleus, PF - polar filament, PS - polar sac, V- posterior vacuole. Bars: Figs. 10, 11, 14 - 0.5 μm, 12,13 - 100 nm



Figs. 15-19. Details of the polar filament. 15 - slightly oblique longitudinal section close to the centre of the polar filament (sloping inwards towards the polar sac), only the centre of the filament unites with the polar sac. The dense material in the external zone of the polar sac (black arrowheads) forms a cylinder-like structure around the zone where the polar filament enters. The anterior pole of the polar filament is shaped like a collar, dark arrows indicate an aperture of the same width as layer 3 of the filament. In the obliquely-sectioned filament the dense material of layer 1 continues into the collar (white arrows). White arrowheads indicate the spiral-wound external ridges of layer 1 (separating the tubules). 16 - slightly oblique section of the collar outside the aperture (sloping outwards towards the polar sac); the anterior component of the collar is continuous with the dense external zone of the polar sac (black arrowheads); white arrowheads indicate traces of spiral-wound dense filament material (layer 1). 17 - transversely-sectioned coil in a microspore, the layers of the core are numbered 1-4; black arrowheads indicate the dense external ridges of layer 1. 18 - aggregated coils in a macrospore; the layers of the filament are numbered as above. 19 - longitudinally-sectioned spore wall with the layers indicated. C - collar, E - endospore, EX - exospore, M - membrane, P - plasma membrane, PS - polar sac, T - tubule. Bars - 100 nm

right angles from a flat central portion (Fig. 15). There was no anchoring disc inside the polar sac (Fig. 15). Longitudinal sections through filament and sac taken through the centre differed from sections taken more peripherally (Figs. 15, 16). The anterior part of the polar filament formed a collar-like structure where two about 8 nm thick electron-dense discs with a diameter of about 220 nm were separated by an approximately equally wide lucent zone. The periphery of the collar was more or less curved backwards (Fig. 16). The unit membrane lining of the polar sac continued around the collar and was obviously also continuous with the membrane covering the tubular layer of the filament (Fig. 16). The anterior component of the dense collar appeared to be a continuation of the internal dense layer (layer 3) of the polar filament, and the aperture was identical to the diameter of layer 3 (Fig. 15). It continued into the dense material below the unit membrane of the polar sac (Fig. 16). The dense material formed a cylinder-like internal structure around the zone where the sac united with the filament (Figs. 15, 16). The posterior component of the collar was continuous with the surface layer (layer 1) of the core (Figs. 15, 16). Only the granular centre of the polar filament entered the polar sac (Fig. 15).

The spore wall had three layers (Fig. 19): an external, uniformly thin electron-dense exospore, measuring about 16 nm, a median lucent endospore layer, which might be slightly thinner above the anchoring apparatus, and an internal plasma membrane about 8 nm thick.

The sporont-derived sac

The sac enclosing the spores was not a sporophorous vesicle of normal microsporidian type but the external zone of the sporont. The original plasma membrane and the internal newly-formed unit membrane (Fig. 6) remained visible also in sacs containing mature spores (Figs. 10, 11), while the cytoplasm between the membranes became more condensed and electron-dense. In some sacs with mature spores the enveloping cytoplasm appeared completely devoid of structures. Fragments of the central cytoplasm and vacuoles remained between the spores in the centre of the sporont (Figs. 10, 11).

DISCUSSION

Cytology

The cytological organization of the organism herein corresponds well with the *Chytridiopsis*-like microsporidia as outlined for example by Richards and Sheffield (1970), Purrini and Weiser (1985) and Beard et al. (1990). However, two cytological deviations are apparent. First, the surface of the polar filaments of the genera *Chytridiopsis*, *Nolleria* and *Steinhausia* (based on *S. brachynema*, not the type species, *S. mytilovum*) are externally covered by a continuous coat of small, membrane-lined chambers, resembling a honeycomb. Transverse and longitudinal sections through this layer look identical. The microsporidium described herein has a system of interwoven tubules below a unit membrane sheath, appearing differently depending on the plane of sectioning (Figs. 14, 17). Second, there are differences in the width of the polar filament. The microsporidium described herein is the first representative of this group of microsporidia with an anisofilar polar filament (wide anteriorly, narrow posteriorly). All other species with known ultrastructural cytology have an isofilar filament, of uniform thickness from pole to pole.

The modification of the sporont in such a way that one cell forms both sporoblasts (from the nucleus and the central cytoplasm) and the spore-containing envelope (from the plasma membrane and the external zone of cytoplasm) (Fig. 8), corresponds with the sporogony of *Chytridiopsis typographi* illustrated by Purrini and Weiser (1985; Fig. 10). The only difference is that the microsporidium examined by us does not lay down an electron-dense cover on the plasma membrane to produce a thick-walled cyst.

Taxonomy

Little is known about the majority of the 21 microsporidian species described from mites. Only three have thus far been investigated at the ultrastructural level. However, the size and shape of the spores clearly differ from the species treated herein, and only two of them need comment.

Only one species, *Nosema steinhausi,* is reported as a parasite of a *Tyrophagus* species (*T. noxius*; see Weiser 1956). As identification of the genus is supported by illustrations of the description, there is no reason to doubt that the species belongs in the diplokaryotic genus *Nosema*. Of the four *Pleistophora* species described by Purrini and Weiser (1981) one, *P. dindali,* is a parasite of the gut epithelium. It produces 2.0-2.5 μm long spores that are described as spherical to oval. None of the four species actually belongs in *Pleistophora*, a genus so far only proven from vertebrates. The extremely brief descriptions give us no information that suggests to what genera they actually belong. However, *P. dindali* is illustrated by one

micrograph of stained spores, and the spores shown there are clearly not spherical.

The uninucleate spherical spores of the microsporidium treated herein exhibit an unusual construction (comparatively short polar filament devoid of a tilted section, no anchoring disc in the polar sac, socket-to-collar attachment of the polar filament, only the central strata of the filament enter the polar sac, no lamellar or sac-like polaroplast, and no polyribosomes) which indicates that this microsporidium is related to the small number of microsporidia united in the order Chytridiopsida Weiser, 1977. With very few exceptions, all develop in the gut epithelium of their hosts. The only reproduction known to occur is the production of sporoblasts. Only a few of these microsporidia are described thoroughly enough so that detailed comparison is possible.

Microsporidia of this construction are classified in 6 genera (disregarding the insufficiently known *Jirovéciana* Larsson,1980): *Chytridiopsis* Schneider, 1884, *Steinhausia* Sprague, Ormières and Manier, 1972, *Hessea* Ormières and Sprague, 1973, *Burkea* Sprague, 1977, *Buxtehudea* Larsson, 1980 and *Nolleria* Beard, Butler and Becnel, 1990.

Hessea squamosa, the only species of the genus, has nuclei coupled as diplokarya and strands of polyribosomes in the spores. The life cycle comprises two sequences of reproduction: merogony and sporogony (Ormières and Sprague 1972). This genus is obviously not related to the other five genera, and it cannot be used for the microsporidium treated herein.

The two species of the genus *Burkea* are probably not closely related, cytological differences speak for that. The type species, *B. gatesi*, has a long polar filament with distinctly tilted coils, and the exospore layer is thick (Puytorac and Tourret 1963). *Buxtehudea scaniae*, the only species of this genus, has a long polar filament with distinctly tilted coils and a characteristic surface layer of the polar filament (Larsson 1980). It is obvious that neither *Burkea* not *Buxtehudea* can accommodate the new microsporidium.

The microsporidia classified in the genera *Chytridiopsis*, *Steinhausia* and *Nolleria* have spores similar to the spores of the microsporidium treated in this description. However, there are two prominent differences: the surface of the polar filament is covered by a characteristic honeycomb-like layer and the polar filament is isofilar (Richards and Sheffield 1970; Purrini and Weiser 1984, 1985; Beard et al. 1990; Larsson 1993). So even if the relationship with these genera is obvious, none of them can be used for the new species, and it is necessary to establish a new genus.

All the recent classifications divide these genera between four families: Chytridiopsidae with *Chytridiopsis*, *Nolleria* and *Steinhausia*, Burkeidae with *Burkea*, Buxtehudeidae with *Buxtehudea*, and Hesseidae with *Hessea* (see for example Canning 1989). As concluded earlier, only Chytridiopsidae contains closely related microsporidia. The family was established by Sprague and colleagues in 1972. The diagnosis stressed the following characteristics: sporogonial plasmodium develops in intimate contact with the host cell nucleus, often causing a deep cup-shaped depression in it, production either of two kinds of cysts (thick-walled and fragile membrane) or only of a membraneous cyst, spores spherical-elliptical, endospore rudimentary or absent, polaroplast rudimentary or absent, polar filament of short to medium length (Sprague et al. 1972). Beard et al. (1990), based on their investigation of *Nolleria pulicis*, emended the diagnosis, and added one character, which is of particular interest when considering the microsporidium treated by us: polar filament (called polar tube) isofilar.

Considering the diagnostic criteria of Chytridiopsidae strictly, our microsporidium cannot be placed in this family; the contact with the nucleus of the host cell is not close, and the polar filament is not isofilar. The last criterion, the isofilar filament, is clearly useful for the characterization of a genus, but more questionable in the diagnosis of a family. There are several examples of families where genera with isofilar polar filaments are mixed with anisofilar ones, for example Thelohaniidae, Pleistophoridae and Tuzetiidae (Sprague et al. 1992). So, if we ignore this criterion, the only important obstacle for assigning our new genus to the family Chytridiopsidae is the lack of an association with the host nucleus. However, while this association has been verified for *Chytridiopsis* (Sprague et al. 1972) and for *Nolleria* (Beard et al. 1990), to our knowledge it has not been observed in *Steinhausia*. We cannot present substantial arguments for the establishment of a new family, and therefore we elect to classify the new genus *Intexta* in the family Chytridiopsidae.

DESCRIPTION

Intexta gen. n.

Diagnosis: reproduction by sporogony only; sporont with isolated nuclei, spores uninucleate; sporogony by vacuolation, surface layer of the sporont persisting as an envelope around spores; sporophorous vesicle absent; spores spherical, polar filament anisofilar, the complete

filament coiled; polar sac devoid of anchoring disc, attaches directly to the anterior coil of the polar filament; surface layer of polar filament with longitudinally-directed tubules; polaroplast absent; exospore thin, electron-dense; endospore well-developed, not reduced above the polar sac; development not closely associated with nucleus of host cell.

Etymology: the name alluding to the tubules winding around the polar filament (Lat. *intexo* = wind around).

***Intexta acarivora* sp. n.**

Sporogony: each sporont produces small, irregular number of spores; dominant sporogony microsporous; small number of macrospores might appear together with microspores.

Spores: microspores 1.32-1.78 µm in diameter, macrospores 1.49-2.33 µm wide; spore wall of microspores 101-136 nm wide, of macrospores up to 145 nm; exospore about 16 nm; polar filament anisofilar with 2-3 coils in microspores (anterior 1-2 wide), up to 9 coils in macrospores (anterior 3-4 wide); coils of microspores individually arranged, in macrospores united in bundles; the electron-dense centre of wide coils 136-221 nm wide, of narrow coils 102-128 nm; nucleus of microspores up to 1.2 µm wide.

Host tissues involved: midgut epithelium.

Type host: the forage mite *Tyrophagus putrescentiae* (Schrank, 1781) (Acari, Acaridae).

Provenance: culture in the Netherlands.

Types: syntypes on slide No. 930831-A2-1.

Deposition of types: in the International Protozoan Type Slide Collection at Smithsonian Institution (Washington, DC).

Etymology: based on the name of the host order and the Latin verb *voro* - eat.

Acknowledgements. The authors are indebted to Mrs. Lina Gefors, Mrs. Birgitta Klefbohm and Mrs Inger Norling, all at the department of Zoology, University of Lund for excellent technical assistance, and to the Alberta Environmental Centre, Vegreville for use of their facilities. The investigation in Sweden was supported by The Swedish Natural Science Research Council and the Royal Physiographic Society in Lund, and in Canada by Alberta Agricultural Research Institute's Farming for the Future Program.

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Received on 16th May, 1997; accepted on 30th July, 1997

Acta Protozoologica (1997) 36: 305 - 309

ACTA
PROTOZOOLOGICA

Sinuolinea indica sp. n. (Myxosporea: Sinuolineidae) Parasitic in the Urinary Bladder of a Sciaenid Fish from the Hooghly Estuary, West Bengal, India

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Summary. A new species of Myxosporea, *Sinuolinea indica* sp. n. (Bivalvulida: Varliisporina: Sinuolineidae) is described from the urinary bladder of an estuarine Sciaenid, *Pseudosciaena coibor* (Ham.) (Pisces: Sciaenidae) of the Bay of Bengal, West Bengal, India. A morphometric comparison of *Sinuolinea indica* sp. n. with its related species is given to establish its distinctiveness.

Key words: estuarine, fish, Myxosporea, *Pseudosciaena coibor* (Ham.), Sciaeneidae, *Sinuolinea indica* sp. n., Sinuolineidae, urinary bladder.

Abbreviations: Pc. - polar capsule, Sp. - spore.

INTRODUCTION

The fish of the family Sciaenidae are common food fish of the Bay of Bengal and the estuary of the river Hoogly in India. So far, only three species of Myxosporea have been recorded from Indian Sciaenids, namely: *Henneguya otolithi* Ganapati, 1941; *Myxidium sciaenae* Sarkar, 1986; *Ceratomyxa daysciaenae* Sarkar and Pramanik,1994.

The present study reveals the presence of three more myxosporean species. One of them, a *Sinuolinea* sp. infecting the urinary bladder of Sciaenid, *Pseudosciaena coibor* (Ham.) is described as a new species.

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MATERIALS AND METHODS

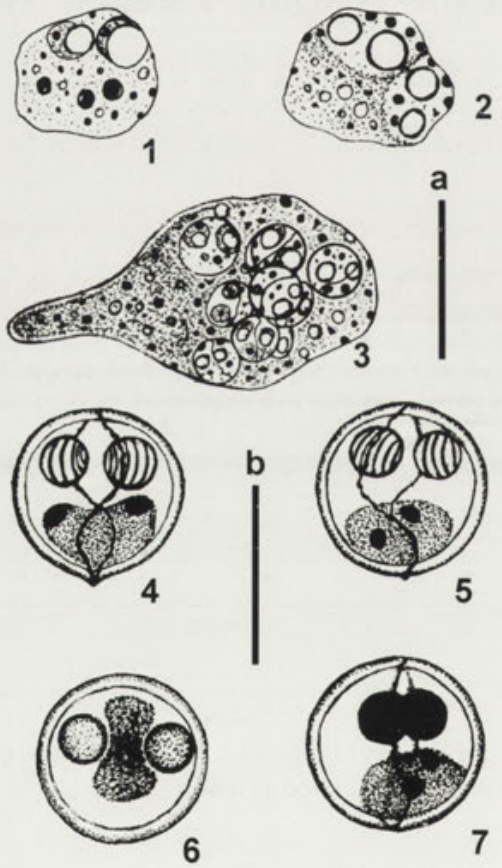
The fishes collected during the months of September to March of 1994-95 and also from September to January of 1995-96, from the landing places of the Hooghly Estuary, were immediately preserved on ice. The hosts were examined thoroughly in the laboratory for the protozoan parasites. These were then studied fresh with Lugol's iodine solution (2%) and on dry smears stained with Giemsa (1:20) after fixation in absolute methanol, under the oil immersion lens. Potassium hydroxide solutions (2-10%) and also saturated solution of urea were used to extrude the polar filament of the spores. The India-ink method (Lom and Vavra 1963) was employed to detect the mucous envelope of the spores.

The measurements were taken by an ocular micrometer from fresh parasites obtained from frozen hosts. The figures were drawn with the aid of a camera lucida.

OBSERVATIONS

Sinuolinea indica sp. n.

Early plasmodium was small and irregular in shape (Fig. 1). A few plasmodia with a pair of developing spores were observed (Fig. 2). The polysporous plasmodia were



Figs. 1-7. Vegetative stages and spores of *Sinuolinea indica* sp. n. 1 - an early plasmodium - Lugol's iodine. 2 - a plasmodium destined to form two spores - Lugol's iodine. 3 - a polysporous plasmodium - Lugol's iodine. 4,5 - fresh spores in sutural view - Lugol's iodine. 6 - a fresh spore in top view, suture and polar filament indistinct - Lugol's iodine. 7 - a spore in oblique view showing partially overlapping polar capsules - Giemsa. Bar a - 20 μm, bar b - 10 μm

elongated triangular with broader anterior surface and narrower posterior surface ending in the form of a lobose pseudopodium (Fig. 3). These forms measured 20.0-58.0 x 13.5-32.0 μm. The free mature spores were spherical in sutural view (Figs. 4, 5) and also in top (Fig. 6) and oblique views (Fig. 7). The suture was slightly ridged (Fig. 4); the sutural line was extremely sinuous, twisted on its axis, often appeared in the form of 8. The two shell valves were thick-walled, smooth and non-appendiculate. The two polar capsules were spherical, equal and usually set wide apart towards the anterior surface (Figs. 4, 6), rarely one capsule being overlapped by the other in oblique view (Fig. 7). There were four to six coils of the polar filament in each capsule. The axis of coiling of the polar filament was almost parallel to the sutural line of the spore which were not discernible in top view (Fig. 6). The spore cavity was partly filled with small mass of binucleated sporoplasm which was restricted to the posterior part of the cavity. There was no iodinophilous vacuole in the sporoplasm and no mucous envelope around the spore.

Measurements (in μm) (n=22) based on spores, treated with Lugol's iodine, from a single frozen host : diameter of spore 10.0-13.5 (12.02±0.74); diameter of polar capsule 3.0-4.0 (3.45±0.35).

Site of infection: urinary bladder (cavity).

Incidence: 4/56 (7.14%).

Pathogenicity: not apparent.

Host: *Pseudosciaena coibor* (Ham.) (Teleostei: Sciaenidae).

Locality: Hooghly Estuary of the Bay of Bengal, West Bengal, India.

Material: syntypes on slide no. Mzs. - 8, deposited to the Department of Zoology, Rishi Bankim Chandra College, Naihati, West Bengal, India.

DISCUSSION

Davis (1917) instituted the genus *Sinuolinea* and transferred *Sphaerospora dimorpha* Davis, 1916 into it which was consequently became the type species. The features such as „trophozoite disporous and polysporous; spores approximately spherical with or without lateral processes, capsules rounded, not convergent when seen from above, capsular pores some distant apart, sometimes on nearly opposite side of the spore; sutural line forming a prominent ridge and twisted on its axis"; were considered diagnostic by Davis (1917). Later Kudo (1919) and Shulman (1966) retained almost the same diagnostic features for *Sinuolinea* Davis, 1917. Lom and Noble (1984) in recent past, proposed the following diagnostic features for *Sinuolinea*: „Monosporous to polysporous trophozoite, spherical to subspherical spore, sutural line

Table 1. Comparison of the related species of *Sinuolinea* Davis, 1917 with *Sinuolinea indica* sp. n.

Parasite (Host)	Trophozoite	Spore	Polar capsule	Infection locus	Locality
Sinuolinea capsularies Davis, 1917 (*Paralichthys albiguttus, P. dentalis, Spheroides maculatus*)	Disporous and polysporous, rounded, 40.0 µm in diam.	Subspherical slightly elongated along the long axis, sutural line much twisted	Large and conspicuous, occupying more than one-half of the spore cavity, coiled filament distinct	UB	Beaufort, U.S.A.
S. dimorpha (Davis, 1916) Davis 1917 (*Cynoscion regalis*)	Disporous and polysporous, very large, 575.0 x 90.0 µm	Subspherical, suture ridged, sinuous around the spore, 15.0 µm in diam.	Large and conspicuous, nearly spherical, 4.5 µm in diam.	UB KT	Beaufort, U.S.A.
S. opacita Davis, 1917 (*Paralichthys albiguttus*)	Disporous, round 22.0 µm in diam.	Nearly spherical with lateral appendages, suture ridged, sinuous around spore	Large, spherical 4.0 µm in diam.	UB	Beaufort, U.S.A.
S. branchiophora Davis, 1917 (*Paralichthys albiguttus*)	Disporous, round to irregular in shape	Central portion nearly spherical with long lateral arm-like appendages, sutural line oblique to long axis, ridged but not twisted, 9.0-11.0 x 9.0 µm	Large, conspicuous, occupying more than one-half of the spore cavity, 3.5 µm in diam.	UB	Beaufort, U.S.A.
S. cyclopterina Basikalowa, 1932 (*Cyclopterous lumpus*)	Polysporous, large, round or irregular, 50.0-60.0 µm	Spherical, slightly elongated, sutural line S-shaped, 14.0-16.0 x 13.0-15.0 µm	Spherical, open on opposite side of the spore, 4.0-5.0 µm in diam.	UB	Barents Sea, White Sea
S. murmanica Basikalowa, 1932 (*Ammodites tobiamus*)	Disporous and polysporous, round or fusiform, 20.0-30.0 µm in diam.	Spherical, sutural line weakly curved, shell valves non-projecting 12.0-14.0 µm in diam.	Spherical, open on opposite sides, 2.0 µm in diam.	UB, UT / KT	Barents Sea

Table 1 (con.)

S. sinuosa Shulman, 1953 (*Boreogadus sida*)	Disporous, round	Spherical, thin-walled sutural line sinuous, not twisted on its axis, 9.0-12.0 µm in diam.	Spherical, open on opposite side of the spore, 3.0 µm in diam.	UB	White Sea
S. magna Yoshino and Noble, 1973 (*Coryphaenoides acrolespis*)	Unknown	Spherical, large, rarely subspherical, suture twisted on its axis, roughly in the figure of 8, configuration extremely variable, sutural ridge distinct, 19.0-30.0 µm in diam.	Spherical, large, well separated at the anterior end, foramina divergent, 7-8 coils of polar filament, extruded filaments twisted at their ends	UB	Mexico, Oregon USA
S. shulmani Gayevskaya and Kovaleva, 1979 (*Zero faber*)	Unknown	Slightly oval, suture extremely sinuous, ends suddenly pointed, 10.6-13.5 x 9.3 - 10.6 µm	Spherical, 2.6 µm in diam.	UB	Atlantic Ocean
S. lesteri Moser, Kent and Dennis 1989 (*Hemiscyllium ocellatum*)	Bluntly rounded at one end and drawn out pointed at the other, 100.0 µm in length.	Slightly elongated in sutural view, sutural line distinct but not ridged, moderately sinuous, 17.0 µm (15.0-19.0 µm) in diam.	Spherical, 3.7 µm (3.0-4.0 µm) in diam., 3-5 coils of polar filament	GB	Heron Island, Australia
S. indica sp. n. (*Pseudosciaena coibor*)	Disporous, triangular, polysporous with lobose pseudopodium, 20.0-25.0 x 13.5-17.0 µm	Spherical, thickwalled, non-appendiculate, suture slightly ridged, sutural line extremely sinuous, twisted on its axis, 12.02 µm (10.0-13.5 µm) in diam.	Spherical, set wide apart, 4-5 coils of polar filament, 3.45 µm (3.0-4.0 µm) in diam.	UB	Hooghly Estuary, West Bengal, India

Abbreviations used: GB - gall bladder, KT - kidney tubule, UB - urinary bladder, UT - ureter.

weakly sinuous or extremely sinuous so that its orientation to the two polar capsules set widely apart, may be difficult to determine; binucleate sporoplasm, coelozoic in the urinary tract of marine fish". As the salient features of the present species clearly conformed with the diagnostic characters of the *Sinuolinea* Davis, 1917, the present species is , therefore, placed to the genus *Sinuolinea*.

Of the *Sinuolinea* spp., the spores of *S. dimorpha* Davis, 1917, *S. opacita* Davis, 1917, *S. capsularis* Davis, 1917, *S. branchiophora* Davis, 1917, *S. cyclopterina*

Basikalowa, 1932, *S. murmanica* Basikalowa, 1932 (both cited from Shulman 1966), *S. sinuosa* Shulman, 1953, *S. magna* Yoshino and Noble, 1973 and *S. lesteri* Moser et al., 1989 show similarity with the spore of the present *Sinuolinea* sp. However, the spores of *S. opacita* and *S. branchiophora* have arm-like lateral appendage while the spore of the present species is non-appendiculate. The other species *S. dimorpha* (Sp. - 15.0 µm, Pc. - 4.5 µm), *S. capsularis* (Sp. - 12.0-14.0 µm, Pc. - 4.5 µm), *S. cyclopterina* (Sp. - 14.0-16.0 µm x 13.0-15.0 µm, Pc. - 4.0-5.0 µm) and *S. magna* (Sp. - 21.2 µm, Pc. - 6.1 µm) clearly show the differences in the dimensions of the spore and the polar capsule of the present species. The spore of *S. murmanica* and *S. lesteri*, although show similarity with the spore of the present *Sinuolinea* sp., the larger spores (12.0-14.0 and 17.0 µm respectively) and smaller polar capsules (2.0 µm of *S. murmanica*) are distinct from the present myxosporean species. Moreover, the sutures of the former two species are weakly sinuous while the suture is extremely sinuous in the spore of the present species. Again, the spore of *S. shulmani* Gayevskaya and Kovaleva, 1979 is almost similar to the spore of the present species. But the oval shape of spore and this similar dimension of polar capsule (2.6 µm) of *S. shulmani* are very distinct from the present species. Furthermore, the spore of *S. sinuosa* is much similar to the present species in shape and size but the thin-walled spore, different nature of sutural line and smaller dimension of spore (9.0-12.0 µm) and polar capsule (3.0 µm) of *S. sinuosa* are different from the present species. Moreover, the present species is obtained from the geographically very distant place. In view of such differences with the related *Sinuolinea* spp. (Table 1), the present myxosporean species is considered as a new species and is designated as *Sinuolinea indica* sp. n. since it is the first record of *Sinuolinea* sp. from India.

Acknowledgements. Author is very thankful to Dr. A. K. Roy, Teacher-in-Charge and Prof. Shaibal Roy Chaudhury, Head, Dept. Zoology of this College for providing necessary laboratory facilities. Thanks are also due to Sri Anupam Mazumder, M. Sc. for statistical calculation. Author is also indebted to Prof. K. Goswami and the authority of the R. B. C. College Computer Centre for computer facilities.

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* Not seen in original.

Received on 29th July,1996; accepted 25th July, 1997

Acknowledgement

The editors wish to acknowledge wish to help the following colleagues who have served as reviewers for one or more manuscripts submitted for publication in our journal in 1997.

André Adoutte
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ACTA PROTOZOOLOGICA

NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY
WARSAW, POLAND
1997
VOLUME 36
ISSN 0065-1583

Polish Academy of Sciences
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ACTA PROTOZOOLOGICA

International Journal on Protistology

ACTA PROTOZOOLOGICA appears quarterly.

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This publication is supported by the State Committee for Scientific Research
Printed at the MARBIS, ul. Kombatantów 60, 05-070 Sulejówek, Poland

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VOLUME 36 NUMBER 4