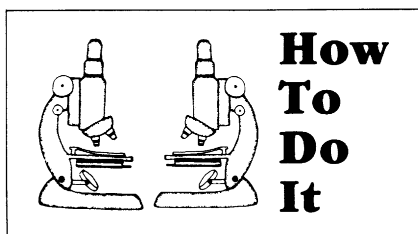

Species Identity of Commercial Stocks of Paramecium *in the U.S.*

Thomas A. Cole Ruchir Sehra Willis H. Johnson

The "aurelia" group of the genus *Paramecium* (Wichterman 1986) contains three major species-complexes of slipper-shaped organisms. *P. aurelia* is generally distinguishable from *P. caudatum* and *P. multimicronucleatum* on the basis of size (120 μm–180 μm length) and its possession of two vesicular micronuclei (approximately 3.5 μm in diameter). Distinguishing between *P. caudatum* and *P. multimicronucleatum* is more difficult because of overlapping size ranges (*P. c.*: 170 μm–300 μm long; *P. m.*: 180 μm–350 μm long). *P. caudatum* has one compact micronucleus (8μm–14μm in diameter) and *P. multimicronucleatum* has between four and eight vesicular ones (0.7 μm–5 μm in diameter). *P. jenningsi* is a more recently described member of the "aurelia" group; it has two micronuclei which are larger than those of *P. aurelia*.

Persons without extensive experience in observing members of these species-complexes of *Paramecium* are inclined to consult *How to Know the Protozoa* (Jahn et al. 1979; Jahn & Jahn 1949). The first revised edition (Jahn & Jahn 1949, p. 188) gives directions for distinguishing these species-groups on the basis of micronuclear staining:

> If acidulated methyl green is added to the slide (of fixed cells), the nuclei will stain. If you can find a micronucleus, the animal is *P. caudatum;* if you examine a dozen well-stained specimens and cannot find the micronucleus then it is probably *P. multimicronucleatum*.

This statement emphasizes the difficulty of positively identifying *P. multimicronucleatum* by simple and rapid staining procedures. The second edition of the *How to Know the Protozoa* (Jahn et al. 1979) provides a similar description for staining, but suggests hematoxylin, rather than methyl green, as the micronuclear stain. A recent study on the binding of fluorescent FITC-concanavalin A to cellular structures in *Paramecium* has shown that considerable variation exists in the size of the micronucleus in different strains (Allen et al. 1988) of *P. multimicronucleatum*. Difficulty with species-complex cultures of *Paramecium* in our and another laboratory (Allen et al. 1988; Fok et al. 1987) led us to explore different staining procedures for distinguishing these larger forms, *P. caudatum* and *P. multimicronucleatum*. These procedures involve the use of DNA-binding, fluorescent compounds. We then tested stocks from 12 commercial sources. The details of the staining procedures and the results are presented in this article.

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Materials & Methods

Stocks

Cultures of *P. caudatum* and *P. multimicronucleatum* were requested from biological supply companies in the summer of 1987. Stocks were purchased from those companies that did not supply samples. For companies that used the same primary source for *Paramecium* stocks, only one test sample was included in the results. The supply houses were:

1. Evergreen Biological Supply, Inc., Lynnwood, WA
2. Blue Spruce Biologicals, Castle Rock, CO
3. Carolina Biological Supply Company, Burlington, NC/Gladstone, OR
4. College Biologicals, Escondido, CA
5. Frey Biologicals, Mansfield, OH
6. W.A. Lemberger, Oshkosh, WI; Parco, Vienna, OH; Kons, Germantown, WI
7. Nasco, Fort Atkinson, WI
8. Nebraska Scientific, Omaha, NE; Connecticut Valley Biological, Southampton, MA
9. Sargent-Welch Scientific, Skokie, IL
10. Southern Biological, McKenzie, TN
11. Triarch, Ripon, WI
12. Ward's Natural Science Establishment, Rochester, NY.

Additionally, axenic stocks of *P. primaurelia, P. tetraurelia, P. caudatum* and *P. multimicronucleatum* and *Klebsiella*-grown cultures of *P. sonneborni* and *P. jenningsi* were used in studies to standardize the tests.

Chemicals

Acridine orange, 1 mg ml^{-1} in 10 percent acetic acid, deionized water or 0.05 phosphate buffer (pH 7.4) (Sigma, St. Louis, MO).

DAPI (4′, 6-Diamidino-2-phenylindole), 10 μg ml^{-1} Sigma, St. Louis, MO

Hoechst 33258 and 33342, 10 μg ml^{-1} (Sigma, St. Louis, MO)

Ethidium bromide, B grade, 25 μg ml^{-1} (Calbiochem, La Jolla, CA).

Lugol's iodine was prepared with 6 g KI and 4 g I_2 in 100 ml water.

Schiff's Reagent A: $NaHSO_3$, 1.3 g; 1 N HCl, 10 ml; H_2O, 200 ml.

Schiff's Reagent B: 1 g basic fuchsin in 100 ml warm, deionized water, filtered through Whatman #1, 20 ml 1 N HCl and 1 g $NaHSO_3$ added.

Schiff's Reagent: Combine A and B together, age for 24 hours, decolorize with Norit and filter through Whatman #1.

Methyl-green: Acidified methyl-green nuclear stain followed the directions for standard technique (Lee et al. 1985).

Fluorescence extender: 1,4-diazobicyclo[2,2,2]-octane (Sigma) was used at a concentration of 25 mg/ml. Citifluor (Citifluor Limited, Northampton

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Square, Connaught Building-Room 303, The City University, London EC1V OHB, England) was also used as a fluorescence extender according to the directions of the supplier of this mountant medium. Note: The DNA-binding dyes should be handled with care, using gloves and proper disposal procedures characteristic of those for use of ethidium bromide.

Cultures

Axenic cultures were maintained on NDF (non-dialyzable fraction) medium (Johnson et al. 1980). Staining procedures were carried out on stocks as received or on subcultures grown on *Klebsiella*-cerophyl medium according to ATCC recipe 802, Sonneborn's growth medium (American Type Culture Collection 1978). *P. sonneborni* and *P. jenningsi* were maintained on ATCC medium 802.

Fixing & Staining

Fixing was with:

1. 67 percent ethanol (0.5 ml cell culture and 1.0 ml 100 percent ethanol)
2. 7.4 percent formaldehyde in 1X phosphate buffer (0.05 M, pH 7.4)
3. Formalin-acetone (0.5 ml cell culture and 1.0 ml 4 percent formalin in phosphate buffer followed by centrifuging and resuspending in 1 ml of acetone) or
4. 2 percent glutaraldehyde in 1X phosphate buffer, pH 7.4.

Fixed cells were washed in phosphate buffer or deionized water. Samples of pelleted cells were either mixed on a slide with the fluorescent dye, or equal volumes of fixed, suspended cells and dye were mixed and transferred to the slide for observation. Samples of stained cells were observed with a Zeiss Standard or an Olympus IMT-2 inverted microscope, each with epifluorescence capability. With the Zeiss microscope, the FITC system (450 nm–490 nm EX, >529 nm barrier filter) was used for acridine orange and ethidium bromide dyes. The ultraviolet system (300 nm–400 nm EX, >420 nm barrier filter) was used with the Hoechst and DAPI dyes. With the Olympus microscope, the B filter set (450 nm–490 nm EX, >515 nm barrier filter) and the UGI filter set (300 nm–405 nm EX, >420 nm barrier filter) were used correspondingly.

Feulgen Procedure

Cells were fixed with Nissenbaum's fluid (Nissenbaum 1953) by dropping the fixative onto a drop of concentrated protists on a slide. Lugol's iodine was then added to the drained slide. This slide was placed in 70 percent ethanol, then hydrated to water in steps of 50 percent, 30 percent and deionized water. Hydrolysis in 1 N HCl was carried out at room temperature for 2 minutes, then at 60 C for 8 minutes and then at room temperature for 2 minutes. It was subsequently incubated in deionized water for 5 minutes and 1 percent formalin for 15 minutes. Rinsing in deionized water was followed by staining in Schiff's reagent for 12 minutes to 15 minutes. A water rinse began the rapid dehydration series in steps of 30 percent, 50 percent, 70 percent, 95 percent and 100 percent ethanol. In some cases the cytoplasm was counterstained during the dehydration procedure with 0.1 percent Fast Green in 90 percent ethanol between the 70 percent and 95 percent steps. Direct observation without a mountant medium followed.

Mycoplasma Testing

Since fluorescent DNA-binding dyes are used to detect mycoplasma inflections, cultures were tested for mycoplasma contamination using a tissue culture detection kit (^{3}H-DNA probe for mycoplasma DNA) with positive and negative controls (Gen-Probe, San Diego, CA 92123). Directions were precisely followed, using 1.0 ml of mid-log phase axenic paramecium culture in place of the 1.0 ml tissue culture fluid called for by the Gen-Probe kit protocol.

Results

Micronuclear Staining of Known Species Groups

Axenic cultures of *P. primaurelia*, *P. tetraurelia*, *P. caudatum*, *P. multimicronucleatum* and *Klebsiella*-cerophyl cultures of *P. jenningsi* and *P. sonneborni* were subjected to each type of fluorescent dye. The proper number of micronuclei was identified in each case: one for *P. caudatum*; two for *P. aurelia* species, *P. jenningsi* and *P. sonneborni*; and more than two for *P. multimicronucleatum*. In three double-blind tests involving 21 samples, the identity of each sample was determined correctly. Use of acidified methyl green stain and the Feulgen procedure was not as reliable. The results of the fluorescent dye staining procedures are independent of the fixing procedure used. Figure 1 shows photographs of three species of *Paramecium* with micronuclei visible as a result of the DAPI stain.

Micronuclear Staining of Commercial Supply House Samples

Of the 12 pairs of samples obtained in 1987 and 1988, five were supplied correctly according to the number and size of the micronuclei (Allen et al. 1988; Wichterman 1986). Six suppliers provided *P. multimicronucleatum* for both species-complexes, *P. multimicronucleatum* and *P. caudatum*. The remaining house supplied a sample of *P. caudatum* with extremely small nuclei (1 μm–3 μm diameter). Two of the supply houses that provided authentic stocks as judged by this study previously had difficulties with the identity of the species-groups in question (Allen et al. 1988; Personal communication with supply company representative, June 1987). Thus, nine of 12 supply companies have had some species-complex identity problems or a micronuclear size aberration with stocks of genus *Paramecium* in the last several years.

Mycoplasma Testing

All the axenic *Paramecium* cultures that were tested for mycoplasma infection by DNA-RNA hybridization were negative. These results showed that the structures labeled with DAPI or the Hoechst dyes were not due to mycoplasma infection.

Discussion

The use of the five fluorescent, DNA-binding dyes has given consistent results for the micronuclear condition of members of four species-complexes (*P. a.*, *P. c.*, *P. j.*, *P. m.*) *P. sonneborni* is the 15th member of the *P. aurelia* complex. This reproducibility, along with the simplicity of the procedure, makes the technique attractive. Acridine orange (Abrams, Diamond & Kane 1983; Royere et al. 1988), as a general cytological stain, lights up many cellular structures, but the micronuclei can be distinguished from other structures that bind this dye. Allen and co-workers (1988) have described analogous results with a fluorescent (FITC) form of the lectin concanavalin A. Cell fixation is not absolutely necessary to visualize the micronuclei with these fluorochromes. Aqueous solutions of the five fluorochrome dyes may be mixed with living cells and mobile cells with fluorescent

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nuclei result, an indication of the simplicity of the use of these fluorochromes. This effect is most pronounced with DAPI. Precise viewing of the micronuclei requires immobilization with a viscous agent (Protoslo, methyl cellosolve) or nickel sulfate (0.03 percent–0.35 percent).

If a microscope is available with UV- or FITC-epifluorescence capability, these small, DNA-binding fluorochromes provide a simple and rapid method for distinguishing between members of the two large species-complexes of *Paramecium*.

It is surprising that eight of the 12 biological supply house samples of *Paramecium caudatum* have been misidentified in recent years as are six of 12 in the current study. One explanation for such widespread misidentification for the two large and common species-complexes is that once an error of identification or randomization of cultures occurs, it is transmitted among supply companies that obtain stocks from one another when local propagation cultures fail. This suggestion is supported by the circumstantial evidence that all the misidentifications detected here have been of the same type: *Paramecium multimicronucleatum* supplied for *Paramecium caudatum*.

All the supply houses were informed of the overall results of this survey, and each was told specifically if its stock of *P. caudatum* was correct. They were not informed of the specific results for other suppliers stocks. The six that supplied *P. multimicronucleatum* for both species-complexes were given a photograph of the stained, aberrant stock and offered an authentic sample of *P. caudatum*, as determined by this study. Some requested an authentic sample; some did not.

Most of the *Paramecium* stocks obtained from biological supply houses probably are used in high school and undergraduate laboratories. In these applications, the size of the organism and its behavior are more important than the identity of the species-complex. However, some commercial catalog descriptions for *P. caudatum* note the large micronucleus or indicate that this species-complex is useful for staining the micronuclei. In these teaching applications, misidentifying the two species complexes (*P. c.* and *P. m.*) would produce unexpected and unexplainable results.

For research purposes, strains of the *Paramecium* species-complexes should be obtained from laboratories that can provide authentic samples or from American Type Culture Collection (12301 Parklawn Dr., Rockville, MD 20852). Even though the cost of a *Paramecium* culture from ATCC is several times that from a biological supply house, the potential for greater expense with the supply house culture is obvious.

For persons using supply house cultures for research work of a publishable nature, the need for correct identification of the cells early on in the work is also obvious. As Allen (1988) has mentioned, identifying micronuclei in *Paramecium* has not always been easy for the casual observer. Techniques for visualizing the micronuclei may be done with the classical Feulgen, methyl green, hematoxylin, etc., stains or with the fluorescent methods described in Allen et al. (1988) or this report. The FITC-concanavalin A method of Allen et al. and acridine orange result in many cellular structures being labeled in addition to the micronuclei, but the micronuclei are clearly discernible.

DAPI, the Hoechst dyes and ethidium bromide bind to DNA and may give positive identification with the simplest of preparation procedures. DAPI staining has been applied to other protists, *Tetrahymena thermophila* (Allis et al. 1987; Wu et al. 1988) and marine gymnamoebae (Rogerson 1988). The requirement of the proper fluorescent-filter system for the different DNA-binding fluorochromes may be a limiting factor. If species-complex identity is a crucial factor and fluorescence capability is not available, the procedures for the classical stains for micronuclear differentiation are included here.

With respect to the small, odd-sized micronucleus in one sample of *P. caudatum*, we note that variation in micronuclear size in *P. multimicronucleatum* is known. Allen and co-workers (1988) found that two stocks of *P. multimicronucleatum* had micronuclei of quite different sizes (2.5, 5.0 μm diameter); that is, one twice the diameter or eight times the volume of the other. Thus, even though the stock of *P. caudatum* in this study clearly is not a case of misidentification, its use for micronuclear staining observations would give unexpected results.

Whether this case of misidentified stocks of genus *Paramecium* is an isolated instance in the U.S. and whether it has occurred in other parts of the world are points of some interest. Workers on other continents as well as in North America might find a check on their maintained stocks to be worthwhile.

The simplicity of the tests described here should aid in verifying the stocks of the species-complexes, if the proper fluorescent-filter microscope is available.

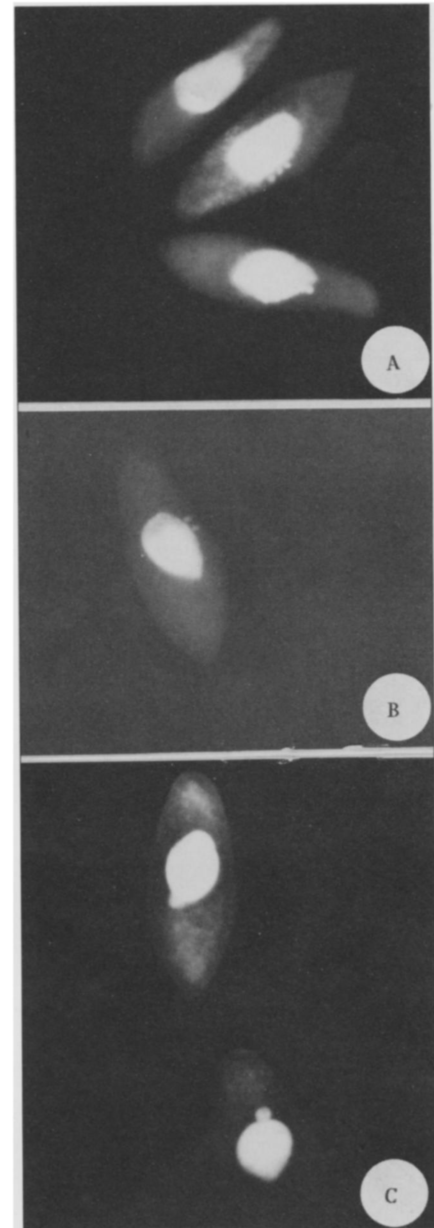

Figure 1. Photomicrographs of A. *Paramecium multimicronucleatum* (ATCC 30725), B. *Paramecium jenningsi*, C. *Paramecium caudatum*. Stained with DAPI, 200x. Kodak 2415 Technical Pan Film, high contrast, speed = 125, for fluorescence, settings at 4x speed (500). Epifluorescence system as indicated in text.

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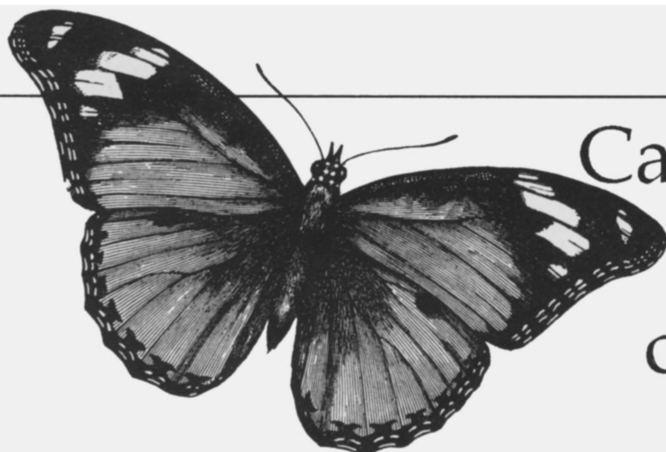

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