with a limited number of *P. multimicronucleatum* and *P. jenningsi* strains.

The large size yet rapid growth rate (four fissions per day at 27°C) of *P. sonneborni* n. sp. allows some consideration of potential uses for this species in research. When observed by phase-contrast optics, rotocompressed cells have easily visible cortical structures, compared with others of the *aurelia* complex. Chatton-Lwoff silver impregnation yields especially good preparations: this characteristic, along with the ease of observing cortical structures in vivo, suggests that the organism could be useful in studies of cortical morphogenesis. Its large size makes it especially promising for studies using microinjection techniques. Finally, the unusually long clonal life-span of the type-strain makes worthwhile a comparative study with other species of the complex that have a much shorter life-span.

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Intraspecies Variability in the Esterases and Acid Phosphatases of Four Species of the *Paramecium aurelia* Complex[1]

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ABSTRACT. One hundred eighty-eight stocks of *Paramecium primaurelia, P. biaurelia, P. tetraurelia,* and *P. octaurelia* were grown axenically and screened for variation in four different esterases and acid phosphatase using starch gel electrophoresis. Major observations: frequency of intraspecies variation for these enzymes is much lower in these four species than in other organisms; hypervariability for two esterases occurs in *P. biaurelia* both in isolates from worldwide locales and in a restricted locale; clustering of variations occurs in a high proportion of variant stocks in all four species; frequency of intraspecies variation is highest in Central and South America for all four species; and geographical differentiation is lacking between stocks in the same species both for common as well as variant phenotypes despite the cosmopolitan distribution of these species. These results are not correlated with adaptations that favor inbreeding over outbreeding, nor is the possession of bacterial endosymbionts strongly correlated with enzyme variation. When the frequency of intraspecies variation was examined for the *aurelia* complex of species as a whole for 13 enzymes, mitochondrial DNA, and ribosomal DNA, differences between enzymes in frequency of variation could be seen, ranging from less than 2% for seven enzymes to 12.4% for glucosephosphate isomerase, a value similar to that observed for malic dehydrogenase, mitochondrial DNA, and ribosomal DNA in *P. tetraurelia*. The percentage of polymorphic enzyme loci in the complex as a whole was found to be much lower than that observed for other organisms. For the species more intensely studied in this paper the level of genetic polymorphism was also much lower, although *P. biaurelia* showed greater variability for two of the enzymes.

GENETIC polymorphism has been shown to be extraordinarily high using the technique of enzyme electrophoresis for identifying genetic variants. Surveys of genetic variation have been carried out in many of the major groups of plants and animals (see 21, 26, and references therein), and almost without exception the amount of genetic polymorphism has been found to be high. It is therefore of special interest when a group of organisms is found to exhibit a low amount of such polymorphism. The *Paramecium aurelia* complex of 14 sibling species (33) appears to be such an exception.

In an earlier communication (1), in which the results of surveying these species for nine different enzymes were examined, we observed two unusual features: stocks within a species showed little variability, with the exception of one species, although there was considerable differentiation between species; and geographical differentiation was lacking between the stocks of any one species with regard to enzyme polymorphism.

These observations, however, were based on only a limited

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number of stocks surveyed in each species, with the exception of *P. primaurelia* and *P. biaurelia*. It was clear to us that a more extensive sampling of stocks was needed so that these features could be examined more closely. Therefore, the present study was initiated in which as many as possible of the wild stocks in four species—collected from many different sites in different parts of the world—were transferred to axenic culture and examined for six different enzymes. The four species chosen were: *P. primaurelia, P. biaurelia, P. tetraurelia,* and *P. octaurelia.* These species differ in their degree of inbreeding, tolerance to various environmental stresses, content of endosymbiotic bacteria, ability to mate across species lines, and geographical distribution (23, 28–30).

The six enzymes included five esterases and acid phosphatase. In most species these enzymes are very polymorphic in all taxa, with frequencies that run two to three times that of the average enzyme (26). The five esterases in the *aurelia* complex can be distinguished on the basis of their substrate specificity, sensitivity to the inhibitor eserine sulfate, and their response to different growth conditions (5, 8, 12). These five esterases are Esterase A (EstA), Esterase B (EstB), Cathodal Esterase C ($EstC_C$), Anodal Esterase C ($EstC_A$), and Esterase D (EstD). Each type varies independently, with both genetic as well as environmental factors determining the electrophoretic mobility or activity of individual esterases (6–8, 11, 13). For each esterase, each species has a commonly observed subtype (of a particular mobility) that may, or may not, be shared with other species (2, 6). With the exception of *P. primaurelia* and *P. pentaurelia,* each species has a distinctive constellation of esterase subtypes. A variant subtype differs in mobility from the common subtype and may have a phenotype that is unique or similar to a form found in a different species (8).

Electrophoretic forms of acid phosphatase have not been described previously for the *P. aurelia* species complex since this enzyme is very tightly bound to membranes in *Paramecium.* Special techniques are needed to free these enzyme forms and to permit their resolution using starch gel electrophoresis. In this paper the techniques for their resolution will be described and the electrophoretic patterns compared among stocks in these four species.

The degree of intraspecies variation for the esterases and acid phosphatases will be examined in each of the four species and the four species compared. We will consider the factors that appear to affect the distribution of the variants and determine whether geographical differentiation of stocks occurs. Using data on 12 enzymes, the amount of genetic polymorphism in the four species and in the *aurelia* complex as a whole will be compared to data on other organisms. In the next communication from this laboratory, species relationships in the *P. aurelia* complex will be updated using 11 enzymes, including acid phosphatase (2).

MATERIALS AND METHODS

Stocks. Tables I–IV list the individual stocks tested in each species along with other relevant information including geographical origin. Most of the stocks were obtained from the Sonneborn collection at Indiana University and now housed at the American Type Culture Collection, Rockville, MD. Cultures of this collection growing on living bacteria were kindly supplied by Myrtle V. Schneller. A few stocks (299vW, 151vW) were obtained from W. J. van Wagtendonk; still others were collected from the Ann Arbor area (see Tables I and II). Cal 1958 and NG-1 were stocks of paramecia unidentified as to species. Stocks growing on living bacteria were transferred to axenic culture by methods described elsewhere (5, 10). Axenic stocks were maintained in Maintenance Medium, which contains the same components as Axenic Medium with the addition of autoclaved bacteria. Details of the culture media have been described elsewhere (10).

Growth of cultures for extracts. All of the stocks were grown on Axenic Medium to the stationary phase (7–12 days, depending on the growth rate of the particular stock) in 3-liter flasks or 1-liter diphtheria toxin bottles (11). Some of the stocks were also tested after growth in Maintenance Medium or Adaptation Medium (10).

Identification of esterases. For enzyme extracts, all cultures were grown at 23°C and harvested by centrifugation; extracts of the cell concentrates were made by freeze-thawing. Electrophoresis of whole-cell extracts was carried out in 12% starch gels using Electrostarch as previously described (3–5, 7). Two substrates were used, α-naphthyl propionate and α-naphthyl butyrate. Details of the histochemical procedures may be found elsewhere (5), and we have also described use of the inhibitor eserine sulfate in an earlier paper (8).

Identification of acid phosphatases. For enzyme extracts, all cultures were grown at 23°C and harvested by centrifugation. Extracts of the cell concentrates were frozen-thawed and could be stored for about three months at −20°C before carrying out the next step without noticeable deterioration of the enzyme. To 100 μl of frozen extract was added 100 μl of detergent solution (20 mM Triton X-100 and 20 mM sodium deoxycholate), and the mixture was vortexed thoroughly over ice. Electrophoresis was carried out in 12% starch gels using Electrostarch and a buffer containing 6.5 mM sodium deoxycholate, 2.5 mM Tris (Sigma 7-9), and 27.5 mM boric acid, pH 7.7, to which 1.5 mM Triton X-100 and a few drops of antifoam were added after cooking and before degassing. The running buffer contained 127 ml of 0.3 M Tris and 672 ml of 0.3 M boric acid, pH 7.7. Gels were electrophoresed for 8 h at 400 V at 4°C. After electrophoresis the gels were trimmed and sliced into sections 3 mm thick, the anodal portions were cut into vertical strips, and the strips were placed into test tubes and incubated in substrate for 30–45 min at 23°C (see 9). The reaction mixture contained the substrate, sodium α-naphthyl acid phosphate (1 mg/ml), and Fast Garnet GBC (4-amino-3:1′-dimethylazobenzene; C.I. 37210) at a concentration of 1 mg/ml in 50 mM sodium acetate buffer, pH 5.0. After incubation, the gel strips were rinsed with distilled water, covered with 7% (v/v) acetic acid, and refrigerated until photographed.

RESULTS

General aspects. Each stock was grown under conditions that would optimize the visualization of the enzymes in the gels (see Materials and Methods). The mobilities of the esterases and acid phosphatases were compared after electrophoresis of extracts and staining of the gels. The esterases of all stocks were tested for substrate preference, and eserine inhibition was examined for at least two stocks having the commonly observed phenotype and for each of the variant stocks to identify the five types of esterases (8). Extracts of each stock were run against extracts of a stock having the commonly observed phenotype for each of the species, and the mobilities of the enzymes were compared. To verify similarity in phenotype, variant stocks that had similar patterns were run against each other and were compared to stocks in other species.

One hundred eighty-eight stocks were examined: 45 from *P. primaurelia* (Table I), 86 from *P. biaurelia* (Table II), 30 from *P. tetraurelia* (Table III), and 27 from *P. octaurelia* (Table IV). Each table gives the phenotype of individual stocks for four (three in the case of *P. tetraurelia*) different esterases and for acid phosphatase. Subtype I represents the common, or nonvariant, phenotype, of each type of enzyme in each species.

TABLE I. *Enzyme subtypes and other characteristics of stocks of* Paramecium primaurelia.[a]

Stock	EstA	EstB	$EstC_C$	$EstC_A$	Acp	GPI (37)	Symbiont (28)	Geographical origin
16	I	I	I		I	I	–	Woodstock, MD
26	I	I	I		I	I		Stanford, CA
41	I	I	I		I			Atlanta, GA
43	I	I	I		I			Stanford, CA
60[b]	I	I	I		II	I		Burlington, VT
61	I	I	I		I			Woods Hole, MA
74	I	I	I		I			Nashville, TN
90	I	I	I		I			Bethayres, PA
103	I	I	I		I			Philadelphia, PA
119	I	I	I		I			Gwynedd, PA
129	I	I	I		I	I		Ft. Lauderdale, FL
143	I	I	I		I	I		Falkirk, Scotland
144	I	I	I		I	I		Chantilly, France
147	I	I	I			I		Sendai, Japan
153	I	I	I		I			New Haven, CT
156	I	I	I		I			New Haven, CT
168	I	I	I		I	I		Sendai, Japan
171	I	I	I		I			Yamagata, Japan
175	I	I	I		I	I		Puno, Peru
177[b]	I	I	III		I			Santiago, Chile
180	I	I	I		I			Tokyo, Japan
181	I	I	I		I			Tokyo, Japan
182	I	I	I		I			Sano-si, Japan
183	I	I	I		I			Tokyo, Japan
192	I	I	I		I			Woods Hole, MA
217	I	I	I		I			Crystal R. Springs, FL
241	I	I	I		I			Thompson R., #70, LA
243	I	I	I		I			Steinhatchee R., FL
244[b]	I	I	III		III			Escofina R., US 90, FL
257[c]	I	I	I		I	II		Cuernavaca, Mexico
258[b]	I	I	I		IV			? , Mexico
285	I	I	I		I			Stanford, CA
313	I	I	I		I			Los Angeles, CA
334	I	I	I		I			Rostov, USSR
335	I	I	I		I			Ashkhabad, USSR
336	I	I	I		I			Clear Lake, MI
337	I	I	I		I			Kamchatka penn., USSR
513[c]	I	I	I		I	III		Chantilly, France
540[b]	I	II	II	7.3–7.4	V	I	μ	? , Mexico
551	I	I	I		I		μ	San Francisco, CA
555	I	I	I		I		μ	Monterey, CA
561[b]	I	II	II		VI		δ	Pisa, Italy
Cal 1958	I	I	I		I			?, CA
NG-1[b]	I	III	I		I			?, India
1st S	I	I	I		I			1st Sister Lake, Ann Arbor, MI

[a] Key to abbreviations: EstA = Esterase A, EstB = Esterase B, $EstC_C$ = Cathodal C esterase, $EstC_A$ = Anodal C esterase (distance in cm), Acp = Acid phosphatase, GPI = Glucosephosphate isomerase. Numbers in parentheses under abbreviations = references to the literature.

[b] Variant for esterases or acid phosphatases.

[c] Variant for GPI.

Variant subtypes for each esterase type and acid phosphatase are designated II, III, etc. for each enzyme in each species.

The five esterases of the *aurelia* complex differ in their substrate specificity, sensitivity to the inhibitor eserine sulfate, and their response to particular growth conditions (5, 8, 11). The A esterases react specifically with the substrate α-naphthyl propionate, are inhibited with 10^{-3}–10^{-4} M eserine sulfate, and display isozymes, the more anodal forms being enhanced by growth in Adaptation Medium or in living bacteria. The B esterases react more strongly with α-naphthyl butyrate than with α-naphthyl propionate and are inhibited by eserine sulfate when the concentration is raised to 10^{-2} M. The cathodally migrating C esterases have a slight preference for α-naphthyl propionate as substrate over α-naphthyl butyrate, are resistant to 10^{-2} M eserine sulfate, and are sensitive to low concentrations of acetate in the growth medium. The anodally migrating C esterases have a substrate and inhibitor profile similar to that of the cathodal C esterases but are not sensitive to acetate in the growth medium. The D esterases prefer α-naphthyl butyrate as substrate over α-naphthyl propionate, are resistant to 10^{-2} M eserine sulfate, and are sensitive to the component TEM-4T (tartaric acid esters of beef tallow monoglycerides) and to high concentrations of acetate in the growth medium. Variant subtypes were identified by their difference in mobility from the common subtype in each species (8). Only four of the five esterases show clear-cut intraspecies variation: esterase A (EstA), esterase B (EstB), cathodal esterase C ($EstC_C$), and anodal esterase C ($EstC_A$). Most of the *P. primaurelia* stocks lack $EstC_A$ and none of the *P. tetraurelia* stocks has this enzyme.

Simple freeze-thawing of whole cell extracts releases acid

TABLE II. *Enzyme subtypes and other characteristics of stocks of* Paramecium biaurelia.[a]

Stock	EstA	EstB	$EstC_C$	$EstC_A$	Acp	GPI (37)	Symbiont (28)	Geographical origin
1	Ia	I	I	6.4[b]	I			Strikersville, NY
4	II	I	I	6.2	I			Counterfeiter's Ledge, NY
5	Ia	I	I	6.4	I		?	Elkridge, MD
7	Ib	I	I	6.2	I		κ	Pinehurst, NC
8	II	I	–	6.2	I		κ	Halethorpe, MD
9	Ia	I	I	6.4	I			Buffalo, NY
11	Ia	I	I	6.4	I		–	Baltimore, MD
12	Ia	I	–	6.4	I			Staunton, VA
21	VI	I	I	6.2	I			Woodstock, MD
23	VIIa	I	I	6.2	I			Williamstown, MA
28	Ia	I	I	6.4	I			Woods Hole, MA
30	Ia	I	I	6.0	I	I		Coates Pond, MD
34	Ia	I	I	4.8	I		?	New Haven, CT
35	II	I	–	6.4	I			West Haven, CT
36	Ia	I	–	6.4	I		κ	Hamden, CT
50	Ia	I	I	6.2	I		κ	?, OR
53	Ia	I	I	6.4	I			Bloomington, IN
71	II	I	I	6.4	I			Mitchell, IN
72	VIII	I	I	6.0	I	I		Pyote, TX
86	Ia	I	I	6.4	I			Millbrook, NJ
88[c]	III	I	–	6.0	II			Jenkintown, PA
91	VIIa	I	–	6.2	I			Willowgrove, PA
93	IV	I	I	6.2	I			Broad Axe, PA
100	Ia	I	I	6.0	I			Marlton, NJ
104	Ia	I	I	6.0	I			Media, PA
114	II	I	I	6.2	I		σ	Bloomington, IN
115	Ia	I	I	5.6	I			Bloomington, IN
122	Ia	I	–	5.4	I			Roanoke, VA
149	Ia	I	I	6.4	I			Withlacoochee R., FL
160	II	I	I	6.2	I			Bemedgi, MN
179	VIIa	I	–	6.4	I	I		Santiago, Chile
185[c]	Ia	I	I	6.4	III			El Tabo, Chile
187[c]	XII	II	II	–	IV			Cove Lake, TN
193	II	I	I	6.2	I	I	?	near Tubingen, Germany
197	II	I	I	6.2	I		α, κ	?, Germany
206	Ia	I	I	6.2	I	I		Oslo, Norway
207	Ia	I	I	6.2	I			Oslo, Norway
208[c]	XI	II	I	6.2	I			Oslo, Norway
234	Ia	I	I	6.2	I	I		Kobe, Japan
235	Ia	I	I	6.0	I		?	Bishop, CA
249	Ia	I	I	6.2	I		κ	Chipola R., FL
259	II	I	I	6.2	I			Sheep Lake, Rocky Mt. Nat'l Pk., CO
260[c]	X	II	I	5.8	I	I		Boulder, CO
291	Ia	I	I	6.4	I	I		Antelias, Lebanon
292	Ia	I	I	6.4	I			Beirut, Lebanon
304	Ia	I	I	6.2	I			Heidelberg, Germany
305[c]	X	II	I	6.2	I			Portal, AZ
310	VIIa	I	I	6.4	I	I	κ	Christchurch, New Zealand
318[c]	X	II	I	6.4	I		?	Mentone, CA
333	Ia	I	I	6.2	I			Vyborg, USSR
339	Ia	I	I	6.4	I	I		Alushta (Crimea), USSR
511	VIIb	I	I	6.0	I		κ	Edinburgh, Scotland
517	VIIb	I	I	6.0	I		κ	Gif, France
526	VIII	I	I	6.0	I		κ	Genoa, Italy
527	VIII	I	I	6.0	I	I	κ	Genoa, Italy
537	VIIb	I	I	6.0	I		κ	Genoa, Italy
560[c]	XII	II	II	–	V			?, Italy
562[c]	II	I	II	–	I	I	α, κ	Milan, Italy
563	IX	I	I	6.0	I		κ	Milan, Italy
564	VIII	I	I	5.8	I		κ	Wexford, Ireland
570	VIII	I	I	5.8	I			Georgia, USSR
576	VIIb	I	I	5.8	I		κ	Norwich, England
1010[c,d]	Ib	I	I	6.0	VI	I		Cove Lake, TN
1035[c]	X	II	I	6.2	I			Sakhalin Island, USSR
1038	VIIb	I	I	6.0	I		κ	Syktyvkar, USSR
1039	VIIb	I	I	6.0	I		κ	Lomonosov, USSR
1041	Ib	I	I	6.0	I		κ	Leningrad, USSR

TABLE II. *Continued.*

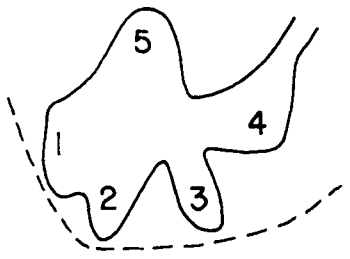
Stock	EstA	EstB	$EstC_C$	$EstC_A$	Acp	GPI (37)	Symbiont (28)	Geographical origin
1042	Ib	I	I	6.0	I			?, USSR
1050[c]	VIIb	I	I	6.0	VII			?, USSR
Hu 35-1	V	I	I	5.8	I			Edinburgh, Scotland
Bl 166-1	Ib	I	I	5.8	I		κ	Edinburgh, Scotland

[a] Key to abbreviations: see Table I. Numbers in parentheses under abbreviations = references to the literature.
[b] Distance in cm from origin.
[c] Variant for esterases or acid phosphatases.
[d] Variant for β-hydroxybutyrate dehydrogenase (HBD; 36).

Ann Arbor Stocks (Botanical Gardens)

Stock	EstA	EstB	$EstC_C$	$EstC_A$	Acp	Collecting site[a] (see also inset figure)
22-1-14	III	I	—	6.0	I	Rattlesnake Pond, Site 1
19	Ia	I	I	6.0	I	Rattlesnake Pond, Site 1
21	Ia	I	I	6.0	I	Rattlesnake Pond, Site 1
28	Ia	I	I	6.0	I	Rattlesnake Pond, Site 1
22-2-12	Ib	I	I	6.0	I	Rattlesnake Pond, Site 2
30	Ia	I	I	6.0	I	Rattlesnake Pond, Site 2
22-3-2	VIII	I	I	6.2	I	Rattlesnake Pond, Site 3
3	Ib	I	I	6.2	I	Rattlesnake Pond, Site 3
4	II	I	I	6.2	I	Rattlesnake Pond, Site 3
5	II	I	I	6.4	I	Rattlesnake Pond, Site 3
6	Ib	I	I	6.2	I	Rattlesnake Pond, Site 3
7	VIII	I	I	6.4	I	Rattlesnake Pond, Site 3
11	VIII	I	I	6.2	I	Rattlesnake Pond, Site 3
22-4-8	VIII	I	I	6.2	I	Rattlesnake Pond, Site 4
33-3	Ib	I	I	6.2	I	Parker Pond

[a] *Rattlesnake Pond:* high grass on edges and duckweed mat nearly complete, path shown with dashed line (see inset figure). Samples from site 5 did not contain paramecia. Site 1 samples also contained *P. primaurelia. Parker Pond* is ca. ¼ mile away from Rattlesnake Pond and may be part of same drainage system.



See Table II (continued).

phosphatases from most of the species in the *Tetrahymena pyriformis* complex (12). This method does not release the acid phosphatases from cells of the *aurelia* complex. Instead, it is necessary to use a combination of detergents to release the enzyme. Moreover, without detergents in the gel, resolution of discrete enzyme forms does not occur and the result is a smear of activity. All of this suggests that the acid phosphatases in *Paramecium* are membrane bound. Triton X-100 apparently dissociates the enzyme and forms a micelle around the enzyme molecule. The micelle contains Triton and a limited number of deoxycholate molecules. When Triton is not added to the gel buffer, the micelle breaks up as migration occurs, resulting in poorly resolved streaks of activity. If Triton is added to the gel buffer, the micelle structure is maintained and resolution and migration improve dramatically. The addition of both Triton and deoxycholate to the gel buffer results in still greater mobility and resolution.

In the four species examined in this paper, there appear to be five zones of activity in the gels. These are labeled A, B, C, D, E, in order of increasing mobility, in the diagrams (Fig. 1). In comparing phenotypes, the pattern of bands was treated as a unit because of the limited mobility and poor resolution of the enzyme forms. Electrophoresis of each extract was repeated at least twice to confirm the phenotype assigned to a stock, and additional extracts were made when assignment was not certain. The pattern observed for *P. primaurelia* and *P. biaurelia* appears to be similar except for minor differences in the intensity of individual isozymes in the doublet found in zone E. This pattern differs from the pattern observed for *P. tetraurelia* and *P. octaurelia,* which appears to be similar. Variant stocks were observed in all four species. Variants were identified by differences in pattern from the commonly observed pattern of the species. The phenotypes of the variants are compared to that of the common subtype in each species in Fig. 1.

Degree of variation within each species. The frequency of intraspecies variation will be examined for each species separately. Then the four species will be compared as to the degree of observable polymorphism.

Paramecium primaurelia. Table I shows that of the 45 stocks examined, seven varied for one or more of the esterases or acid phosphatases (Acp). Four of them—60, 177, 258, NG-1—were variants for one enzyme, one—244—varied for two enzymes, and one—561—varied for three enzymes. Stock 540 varied for the same three enzymes as 561 but had, in addition, an observable $EstC_A$. With respect to particular enzymes, no variants were observed for EstA. Variant stocks were observed for EstB (3/45), $EstC_C$ (4/45), and Acp (5/44), with the frequencies given in Table V.

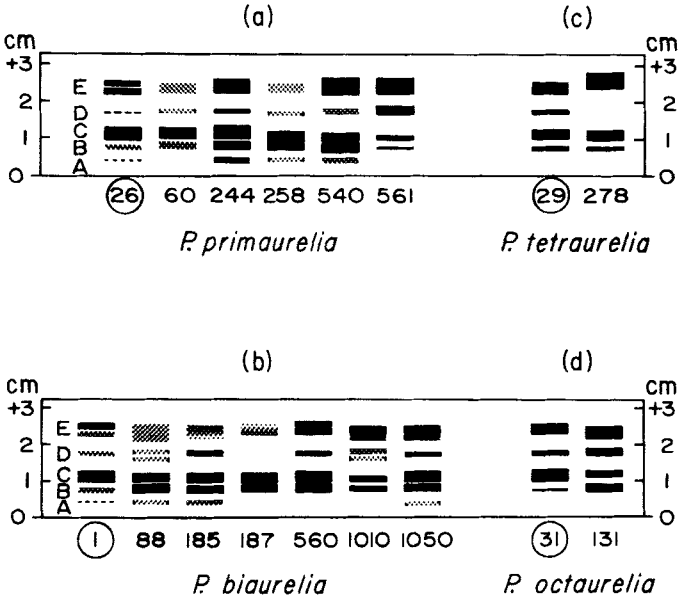


Fig. 1. Comparison of the acid phosphatases in stocks with common and variant subtypes in four species: *Paramecium primaurelia*; *P. biaurelia*; *P. tetraurelia*; *P. octaurelia*. Stocks with the commonly observed subtype, I, are circled. Letters A, B, C, D, E refer to zones of enzyme activity observed in gels. The relative intensities of the bands are indicated by the intensity of shading. Dashed lines indicate bands that are extremely faint and are not always seen on the gels. Distances of migration are marked off in centimeters from the origin (O) on the margins.

This species is cosmopolitan in its geographic distribution, having been collected in North, South and Central America, Hawaii, Asia, and Europe. Table VI details the geographical distribution of the stocks used in this study. Stocks that showed the common subtype, I, for EstA, EstB, $EstC_C$, and Acp were collected from 30 different geographic areas, including 10 different states from the U.S.A., Mexico, Peru, Scotland, U.S.S.R., France, and Japan. Only two stocks were collected from the same site: 180 and 181.

The variant stocks were collected from widely different locations: Vermont, Florida, Chile, Mexico, Italy, and India. Stocks 258 and 540 were both collected from Mexico in 1956, but the records are incomplete as to whether the same or a different collecting site was involved. Table VII gives the worldwide distribution of the variant stocks, and includes two additional stocks variant for GPI. Note that the frequency of variants is more than four times higher for stocks collected outside the U.S.A.,

TABLE III. *Enzyme subtypes and other characteristics of stocks of* Paramecium tetraurelia.[a]

Stock	EstA	EstB	$EstC_C$	Acp	GPI (37)	MDH (38)	mtDNA (16, 22)	rDNA (15)	Symbiont (28)	Cu (24)	Trichocyst discharge (25)	Geographical origin
29[c]	I	I	I	I	I	II				S	+	Hebbville, MD
32	I	I	I	I	I	I			–	S	+	Towson, MD
47	I	I	I			I		I	κ	S	±	Berkeley, CA
51	I	I	I	I		I	I	I	κ, π	S	+	Spencer, IN
111	I	I	I	I						S	+	Baltimore, MD–Washington, DC
126	I	I	I							S	± (ts)	Richmond, FL
127[c]	I	I	I				I	II		S	+	Richmond, FL
139	I	I	I	I					κ, π	S	–	Ft. Lauderdale, FL
146	I	I	I	I	I	I				S	+/–	Honshu, Japan
148	I	I	I	I			I	I		S	+/–	Honshu, Japan
151vW[b]	I	I	I	I								Bok Tower, FL
163	I	I	I	I	I	I				S	–	Valley Forge, PA
170	I	I	I	I		I				S	+	Yamagata, Japan
172[c]	I	I	I	I	I	I	II	I		S	+	Machu Picchu, Peru
173[d]	I	II	I	I						S	+	El Tabo, Chile
174[c,d,e]	I	II	I	I		III				R	+	Valparaiso, Chile
203	I	I	I	I		I	I	I		R	–	Philadelphia, PA
230	I	I	I	I	I					S	+	Sydney, Australia
239[d]	I	III	I	I					λ	S	+	Chocktawatshee R., FL
242	I	I	I	I						S	–	Alabama R., FL
277	I	I	I	I					κ	S	+	St. Mark's R., FL
278[d]	I	I	I	II						S	+	Little Manatee R., FL
280	I	I	I	I	I					S	+	Rappahannock R., VA
298	I	I	I	I					κ	S	+	Empire Range, Panama
315	I	I	I	I	I					S	+	Carrera, Italy
316	I	I	I	I	I			I		S	+	Amsterdam, Holland
322[b]	I	I	I	I								Barro Colorado Island, Panama
329	I	I	I	I						S	+	Cracow, Poland
A1	I	I	I	I					κ, δ			?, Australia
A2	I	I	I	I					κ, δ			?, Australia

[a] Key to abbreviations: see Table I; and MDH = Malic dehydrogenase, mtDNA = Mitochondrial DNA, rDNA = Ribosomal DNA, Cu = Copper sensitivity (S) or resistance (R). Numbers in parentheses under abbreviations = references to the literature.

[b] *Not P. octaurelia.*

[c] Also UV-sensitive, variant for β-hydroxybutyrate dehydrogenase (36; and Smith-Sonneborn, personal communication).

[d] Variant for esterases or acid phosphatases.

[e] Variant for GPI, MDH, mtDNA, or rDNA.

TABLE IV. *Enzyme subtypes and other characteristics of stocks of* Paramecium octaurelia.[a]

Stock	EstA	EstB	$EstC_C$	$EstC_A$	Acp	GPI (37)	Symbiont (28)	Geographic origin
31	I	I	I	I	I		—	Baltimore, MD
130	I	I	I	I	I	I	μ	Ft. Lauderdale, FL
131[d]	I	II	I	II	II		μ, δ	Ft. Lauderdale, FL
137	I	I	I	I	I			?, FL
138	I	I	I	I	I		μ	?, FL
140	I	I	I	I	I			?, FL
141	I	I	I	I	I			?, FL
142	I	I	I	I	I			?, FL
150	I	I	I	I	I			Cypress Gardens, FL
151[b]	I	I	I	I	I			Bok Tower, FL
202	I	I	I	I	I	I		Jal, NM
213	I	I	I	I	I			Miabba R., FL
214	I	I	I	I	I		γ	Waccasassa R., FL
216	I	I	I	I	I		λ	Manatee R., FL
218	I	I	I	I	I	I		Crystal R. Springs, FL
224	I	I	I	I	I	I		Lake Francis, FL
229	I	I	I	I	I		λ	Orange Lake, FL
252	I	I	I	I	I			Tenholloway R., FL
276	I	I	I	I	I	I		Neches R., TX
281	I	I	I	I	I	I		Edisto R., SC
299vW[c]	I	I	I	I	I		λ	Barro Colorado Island, Panama
299I[c,d,e]	II	I	II	I	I	II	λ	Barro Colorado Island, Panama
300[d]	II	II	I	I	I	I		Barro Colorado Island, Panama
307	I	I	I	I	I	I		Canal Zone, Panama
327	I	I	I	I	I		λ	Deland, FL
330	I	I	I	I	I	I		Chatham Co., GA
565	I	I	I	I	I	I	γ	?, Uganda

[a] Key to abbreviations: see Table I. Numbers in parentheses under abbreviations = references to the literature.
[b] From ATCC.
[c] vW = van Wagtendonk; I = Indiana.
[d] Variant for esterases or acid phosphatases.
[e] Variant for GPI.

and that 4/5 stocks collected in Central and South America were variant.

Paramecium biaurelia. Table II shows the results of testing 86 stocks for four esterases and acid phosphatase. Of the 86 stocks, 15 of them were collected from the same or nearby sites in a pond at the University of Michigan's Botanical Gardens in Ann Arbor.

The first point to note is hypervariability for two of the enzymes. Some 12 different phenotypes (I–XII) could be distinguished for esterase A. The most common subtype is I (a + b) found in 41 of the 86 stocks; however, 11 other phenotypes were observed among the other 45 stocks. This hypervariability extends even to stocks collected from the same pond, as illustrated by the Ann Arbor stocks that display four different EstA subtypes. Eight different phenotypes are observed among the 86 stocks for $EstC_A$, including a group of three stocks—187, 560, 562—for which no enzyme activity could be demonstrated. The most common subtypes are I (migration to 6.4 cm; 21/86 stocks), II (migration to 6.2 cm; 29/86 stocks), and III (migration to 6.0 cm; 24/86 stocks). All three of these subtypes are observed among the Ann Arbor stocks. Among the 86 stocks, the distribution of the $EstC_A$ phenotypes appears to be uncorrelated with their EstA phenotype (Chi square = 8.346; df = 5; $P = 0.1$–0.2).

Table II also shows that 12 of the 86 stocks were variant for one or more of the three other enzymes (EstB, $EstC_C$, and Acp). Ten were variant for one of the enzymes—88, 185, 208, 260, 305, 318, 562, 1010, 1035, 1050—and two—187, 560—were variant for all three enzymes. Stock 1010 also varied for HBD (36). With respect to particular enzymes, 7/86 stocks varied for EstB, 3/78 for $EstC_C$, and 6/86 for Acp, with the frequencies given in Table V.

This species is found in moderate to cold climates, having been collected in North and South America, Europe, the Middle East, Asia, and New Zealand. Table VI details the geographic distribution of the stocks used in this study. Twenty-four stocks that showed the common subtype (I) for EstA, EstB, $EstC_C$, Acp,

TABLE V. *Frequency of variant stocks for four esterases and acid phosphatase in four species of the* aurelia *complex.*

	Esterase A		Esterase B		Esterase C_C		Esterase C_A		All esterases		Acid phosphatase		All 3-5 enzymes	
Species	Nos.	%	Nos.	%	Nos.	%	Nos.	%	Nos.	%	Nos.	%	Nos.	%
P. primaurelia	0/45	0	3/45	6.7	4/45	8.9	—	—	5/45	11.1	5/44	11.4	7/45	15.5
P. biaurelia	hypervariable		7/86	8.1	3/78	3.8	hypervariable		8/86	9.3	6/86	7.0	12/86	14.0
P. tetraurelia	0/30	0	3/30	10.0	0/30	0	—	—	3/30	10.0	1/27	3.7	4/30	13.3
P. octaurelia	2/27	7.4	2/27	7.4	1/27	3.7	1/27	3.7	3/27	11.1	1/27	3.7	3/27	11.1
All stocks	2/102	2.0	15/188	8.0	8/180	4.4	1/27	3.7	19/188	10.1	13/184	7.1	26/188	13.8

TABLE VI. *Geographical distribution of stocks in four species of the* aurelia *complex.*[a]

Origin	Species			
	P. primaurelia	*P. biaurelia*	*P. tetraurelia*	*P. octaurelia*
United States				
Vermont	60			
Connecticut	153, 156	34, 35, ***36***		
Massachusetts	61, 192	23, 28		
New York		1, 4, 9		
New Jersey		86, 100		
Pennsylvania	90, 103, 119	88, 91, 93, 104	163, 203	
Maryland	16	5,***8***, 11, 21, 30	29, 32, 111	31
Minnesota		160		
Michigan	336, 1[st] S	22-1-14, 19, 21, 28; 22-2-12, 30; 22-3-2, 3, 4, 5, 6, 7, 11; 22-4-8; 33-3		
Indiana		53, 71, ***114***, 115	***51***	
Virginia		12, 122	280	
North Carolina		***7***		
Tennessee	74	187, ***1010***		
South Carolina				281
Georgia	41			330
Alabama			242	
Louisiana	241			
Florida	129, 217, 243, 244	149, ***249***	126, 127, ***139***, 151, ***239***, ***277***, 278	***130***, ***131***, 137, ***138***, 140, 141, 142, 150,151ATCC, 213, ***214***, ***216***, 218, 224, ***229***, 252, ***327***
Colorado		259, 260		
Arizona		305		
New Mexico				202
Texas		72		276
Oregon		***50***		
California	26, 43, 285, 313, ***551***, ***555***, Cal 1958	235, ***318***	***47***	
Central and South America				
Mexico	257, 258, ***540***			
Panama			***298***, 322	***299vW***, ***2991***, 300, 307
Peru	175		172	
Chile	177	179, 185	173, 174	
Europe				
Ireland		***564***		
Scotland	143	***511***, ***Hu35-1***, ***Bl 166-1***		
England		***576***		
Norway		206, 207, 208		
USSR	334, 335, 337	333, 339, ***570***, ***1035***, ***1038***, ***1039***, ***1041***, 1042, 1050		
Holland			316	
France	144, 513	***517***		
Germany		193, ***197***, 304		
Poland			329	
Italy	***561***	***526***, ***527***, ***537***, 560, ***562***, ***563***	315	
Africa				
Uganda				***565***
Asia				
Lebanon		291, 292		
India	NG-1			
Japan	147, 168, 171, 180, 181, 182, 183	234	146, 148, 170	
Australia			230, ***A1***, ***A2***	
New Zealand		***310***		

[a] Stocks underlined once are variant for EstA, EstB, $EstC_C$, $EstC_A$, or Acp. Additional stocks variant for GPI, MDH, mtDNA, or rDNA are underlined twice. Bold italic indicates symbionts present.

TABLE VII. *Worldwide distribution of variant stocks in four species of the* aurelia *complex.*[a]

Species	Origin				
	United States	Central and South America	Europe	Asia, Australia, Africa	Non-USA
P. primaurelia	2/25 (8%)	4/5 (80%)	2/7 (28.6%)	1/8 (12.5%)	7/20 (35%)
P. biaurelia	6/38 (15.8%) 6/53[b] (11.3%)	1/2 (50%)	5/27 (18.5%)	0/4 (0%)	6/33 (18.2%)
P. tetraurelia	4/16 (25%)	3/5 (60%)	0/3 (0%)	0/6 (0%)	3/14 (21.4%)
P. octaurelia	1/22 (4.5%)	2/4 (50%)	0/0 (0%)	0/1 (0%)	2/5 (40%)
Total	13/101 (12.9%) 13/116[b] (11.2%)	10/16 (62.5%)	7/37 (18.9%)	1/19 (5.3%)	18/72 (25%)

[a] Includes all variant stocks underlined from Table VI.
[b] Includes Ann Arbor stocks.

and $EstC_A$ I and II, were collected from 11 different states from the U.S.A., Norway, Germany, U.S.S.R., Lebanon, and Japan. Only two stocks appear to have been collected from the same site—206 and 207—in addition to the Ann Arbor stocks.

With respect to the 11 other EstA subtypes, there is no real consistency found for the distribution of particular subtypes. Some stocks isolated near each other are similar in phenotype (e.g., 526 and 527 from Genoa, Italy). However, stocks isolated from the same area and even the same pond may be in different groups (e.g., 206 and 208 from Oslo, Norway; Ann Arbor stocks). Also, stocks in the same grouping may be found in widely separated areas (e.g., 305 from Portal, Arizona and 1035 from Sakhalin Island, U.S.S.R.). Similar arguments can be made for the geographical distribution of particular $EstC_A$ subtypes.

Stocks variant for EstB, $EstC_C$, or Acp were collected from widely different locations: Pennsylvania, Tennessee, Colorado, Arizona, California, Chile, Norway, Italy, and the U.S.S.R. Only two of the stocks—187 and 1010—were collected from a similar location, Cove Lake, Tennessee, but presumably not the same site. Table VII gives the distribution of these 12 stocks worldwide where it can be seen that a similar frequency of variants was observed for the U.S.A. and Europe. Of the two stocks collected in South America, one was a variant.

Paramecium tetraurelia. Table III shows that of the 30 stocks examined, three varied for EstB—173, 174, 239—and one varied for Acp—278. The EstB variants are similar in mobility to the common or variant subtypes found in *P. octaurelia* (8). Stock 174 also varies for four other characteristics: it is variant for HBD and MDH, it is resistant to copper, and it is UV-sensitive (24, 36, 38; Smith-Sonneborn, personal communication). No variant stocks were observed for EstA and $EstC_C$. No observable $EstC_A$ is found in any of the stocks. The frequencies of variant stocks is given in Table V.

This species is found in temperate and subtropical climates, having been collected in North, South and Central America, Australia, Asia, and Europe. Table VI details the geographical distribution of the stocks used in this study. Stocks that share the common subtype, I, for EstA, EstB, $EstC_C$, and Acp were collected from 23 different geographic areas, including seven states in the U.S.A., Panama, Peru, Holland, Poland, Italy, Japan, and Australia. A few of the stocks were collected from the same sites (146 and 148 from Honshu, Japan; 126 and 127 from Richmond, Florida, and possibly A1 and A2 from Australia).

The variant stocks were collected from two locations in Florida and two locations in Chile. Table VII gives the worldwide distribution of the variant stocks, and includes three additional stocks variant for MDH, rDNA, or mtDNA. Note that the frequency of variant stocks is similar for collections in the U.S.A. vs. the rest of the world, but that 3/5 stocks collected in Central and South America were variant.

Paramecium octaurelia. Table IV shows that of the 27 stocks examined, two—299I and 300—varied for two of the esterases and one—131—varied for two of the esterases and acid phosphatase. Stock 299I also varied for GPI. Variant stocks were observed for all four esterases and acid phosphatase: EstA (2/27), EstB (2/27), $EstC_C$ (1/27), $EstC_A$ (1/27), and Acp (1/27), with the frequencies given in Table V.

This species is more limited in its geographical distribution, and stocks have been collected from the tropical and subtropical Americas and Uganda, as shown in Table VI. Stocks that share the common subtype, I, for EstA, EstB, $EstC_C$, $EstC_A$, and Acp were collected from 20 different geographic areas, including six states in the U.S.A., Panama, and Uganda. At least four of the stocks—137, 140, 141, 142—were collected from the same site in Florida. One of the variant stocks—131—was collected in Ft. Lauderdale, Florida, while the other two variant stocks—299I and 300—were both collected on Barro Colorado Island, Panama.

Table VII gives the distribution of the variant stocks worldwide. Note that the frequency of variants is almost 10 times higher for stocks collected outside the U.S.A., and that 2/4 stocks collected in Central and South America were variant.

Comparison of variation among species. Twenty-six stocks in all four species varied for one, or more, of the four esterases or acid phosphatase. Ten out of the 26 stocks (38.5%) varied for more than one enzyme, with four stocks varying for two enzymes, five stocks varying for three enzymes, and one stock varying for one esterase plus four additional characters, including two enzymes. All four species contained stocks variant for more than one enzyme, ranging from 25% of the variant stocks in *P. biaurelia* or *P. tetraurelia* to 100% of the variant stocks in *P. octaurelia.* Thus, multiple variations are a common occurrence and a feature shared by all four species.

The frequency of variant stocks for the pooled data from all four species was 13.8% (Table V). This pooled frequency includes stocks that varied for three (*P. primaurelia, P. biaurelia*), four (*P. tetraurelia*) or five different enzymes (*P. octaurelia*), and was similar in all four species (Chi square = 0.246; df = 2; P = 0.7–0.9). However, it should be noted that two enzymes in *P. biaurelia* are hypervariable and were not included in the data used to calculate the frequency for this species.

If individual enzymes are examined, similar but low frequencies of variant stocks are observed in different species for EstB, $EstC_C$, and Acp. For EstA there is greater variation between species, from no variants being observed in *P. primaurelia* and *P. tetraurelia* to hypervariability in *P. biaurelia.* For $EstC_A$

two of the species lack this enzyme, with hypervariability being observed for *P. biaurelia*. The hypervariability seen in *P. biaurelia* for these two enzymes is even observed for stocks in the same pond.

Three of the four species are widely distributed geographically (Table VI). Only *P. octaurelia* is more restricted in its distribution. However, for all four species, stocks that are similar in phenotype were not necessarily collected from nearby sites. In some cases, similar sites are involved. Yet, many of the stocks having commonly observed phenotypes as well as stocks with variant phenotypes were collected from sites widely separated geographically. In general, a much wider distribution of sites is found for the 26 variant stocks than expected on the basis of the distribution of the 162 stocks with commonly observed phenotypes. The latter are distributed among seven, fourteen, eight, and three countries, and four, four, four, and two continents for *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. octaurelia*, respectively. The variant stocks for each of these species, in order, were collected from five, five, two, and two countries, and four, three, two, and one continents.

The distribution of variant stocks worldwide is shown in Table VII. This table includes five additional stocks variant for GPI, MDH, rDNA, or mtDNA. The first point to be noted is that the frequency of variant stocks for two of the species, *P. primaurelia* and *P. octaurelia*, is 4–10 times higher in the collections outside the U.S.A., whereas the other two species, *P. biaurelia* and *P. tetraurelia*, seem to have similar frequencies within and outside the U.S.A. The second point to be noted is the high frequency of variant stocks in all four species collected from Central and South America. Indeed, 10/16 (62.5%) of all the stocks collected in this area are variants. Pooling the data from all four species, the difference in the frequencies of the variant stocks in different areas of the world is significant (Chi square = 5.86; df = 1; $P < 0.05$).

DISCUSSION

Genetic polymorphism and geographical differentiation in the four species. Two features of unusual interest characterized earlier data on intraspecies variation of enzymes in the *P. aurelia* complex: stocks within a species showed little variability, with the exception of *P. biaurelia*; and geographical differentiation was lacking between stocks of any one species with regard to enzyme polymorphism (1). Do these observations stand up on more extensive sampling of the stocks in four of the species?

The major observations of this paper were the following:

(a) The frequency of intraspecies variation for four esterases and acid phosphatase is much lower in these four species than in other organisms, with the exception of two esterases in *P. biaurelia*.

(b) Hypervariability for two esterases occurs in *P. biaurelia* in isolates both from worldwide locales and from a restricted locale.

(c) Clustering of variations occurs in a high proportion of variant stocks in all four species.

(d) The frequency of intraspecies variation is highest in Central and South America for all four species.

(e) Geographical differentiation is lacking between stocks in the same species both for common as well as variant phenotypes despite the cosmopolitan distribution of these species.

These results were surprising because the four species differ in a number of ways. First, they differ in adaptations that favor inbreeding or outbreeding. Sonneborn (31) classified *P. tetraurelia* and *P. octaurelia* as "strong inbreeders" on the basis of their lacking an immaturity period, their short maturity period, and their system of mating type inheritance that favors inbreeding. In both species there is considerable karyotypic diversity and high mortality in crosses between different natural populations. *P. octaurelia*, with a more restricted distribution, would be considered even more of an inbreeder than *P. tetraurelia*. *P. primaurelia* and *P. biaurelia* were classified as "less extreme outbreeders" on the basis of their longer immaturity and maturity periods, although variation between stocks within each species was noted in the length of the immaturity period (31). These two species differ in their system of mating type inheritance, with *P. primaurelia* having a system that encourages more outbreeding. Nyberg (23) speculated that inbreeding is an adaptation that enables closer genetic tracking of environmental changes. In support of his thesis, he found higher tolerance to environmental stress in outbreeding species, such as *P. primaurelia* and *P. biaurelia*, than for inbreeding species, such as *P. tetraurelia*. In addition to the pattern of higher tolerance to stress in the more outbreeding species, there was a tendency toward more variability among the stocks of the inbreeding species. Thus, we might have expected a greater degree of enzyme variability for the inbreeding species, *P. octaurelia* and *P. tetraurelia*, than for the outbreeding species, *P. primaurelia* and *P. biaurelia*. Instead, we find that the degree of variability is low but similar in all four species, but higher in *P. biaurelia* for two of the enzymes. The latter observation supports Sonneborn's (32) finding of greater diversification of the stocks within *P. biaurelia* than for any of the other known species of the *aurelia* complex.

The four species also differ in their content of endosymbionts in the laboratory stocks (27). Stocks known to carry symbionts are identified in Table VI, the specific endosymbiont being noted in Tables I–IV. One or more stocks from all four species carry *delta*; *mu* is found in *P. primaurelia*, *P. biaurelia*, and *P. octaurelia*; *lambda* in *P. tetraurelia* and *P. octaurelia*; *kappa* in *P. biaurelia* and *P. tetraurelia*; *nu*, *sigma*, and *alpha* only in *P. biaurelia*; and *gamma* only in *P. octaurelia* (see Table 1 in Ref. 27 for the binomial names of these bacterial endosymbionts). Six different species of symbionts are found in *P. biaurelia*, four in *P. octaurelia*, three in *P. tetraurelia*, and one in *P. primaurelia*. If the frequency of stocks known to carry symbionts is related to numbers of possible kinds carried by a *P. aurelia* species, we would expect the frequencies of endosymbiont-bearing stocks to be ordered, as follows: *P. biaurelia* > *P. octaurelia* > *P. tetraurelia* > *P. primaurelia*. In the stocks used in this study (excluding the Ann Arbor stocks), the frequencies were: *P. biaurelia*—37%, *P. octaurelia*—37%, *P. tetraurelia*—27% and *P. primaurelia*—9%. What about stocks variant for enzymes compared to stocks with the commonly observed phenotype in terms of endosymbiont content? The frequencies of endosymbiont-bearing stocks having variant and common subtypes were similar in *P. biaurelia* and *P. tetraurelia* (33 and 37% for *P. biaurelia*; 25 and 27% for *P. tetraurelia*), while in *P. octaurelia* and *P. primaurelia* the frequency of variant stocks with symbionts (67% and 29%, respectively) was higher than the frequency of stocks having common subtypes with symbionts (33% and 5%, respectively). Thus, a slightly higher, or similar, frequency of endosymbiont-bearing stocks is observed among those variant for enzymes. However, the case for any association between enzyme variability and endosymbiont diversity falls apart for *P. biaurelia*.

These results were also surprising in view of the fact that other characters are known to vary (24, 25, 32). Moreover, in natural populations of several species, polymorphism for the antigen loci has been observed (29, 30).

P. biaurelia is exceptional in that it possesses a substantial amount of genetic variability for certain enzymes, not only in stocks far apart, but also collected from the same pond. The latter was observed for the pond in Ann Arbor and for Heather-

TABLE VIII. *Frequency of variant stocks for 13 enzymes, mtDNA, and rDNA in 12 species of the* aurelia *complex.*[a,b]

Species	EstA (Refs. 4, 5, and present paper)	EstB	$EstC_C$	$EstC_A$	Acp (2, present paper)	Fum (36)	GDH (36)	HBD (36)	IDH_S (36)	IDH_M (36)	SDH (36)	GPI (37)	MDH (38)	mt-DNA (16, 22)	rDNA (15)
P. primaurelia	0/45	3/45	4/45	—	5/44	0/100	0/100	0/100	0/100	0/100	0/100	2/16		0/2	
P. biaurelia	hyper-variable	7/86	3/78	hyper-variable	6/86	0/110	0/110	1/110	1/110	1/110	0/110	0/16			
P. triaurelia	0/10	0/10	0/10		0/4	0/3	0/3	0/3	0/3	0/3	0/3	1/6			
P. tetraurelia	0/30	3/30	0/30	—	1/27	0/6	0/6	1/6	0/6	0/6	0/6	0/13	2/11	1/6	1/7
P. pentaurelia	0/7	0/7	0/7		0/3	0/2	0/2	0/2	0/2	0/2	0/2	1/9			
P. sexaurelia	0/5	0/5	0/5		0/1	0/3	0/3	0/3	0/3	0/3	0/3	3/8			
P. septaurelia	0/4	0/4	0/4		0/3	0/3	0/3	0/3	0/3	0/3	0/3	2/4			
P. octaurelia	2/27	2/27	1/27	1/27	1/27	0/3	0/3	0/3	0/3	0/3	0/3	1/12			
P. novaurelia	0/5	0/5	0/5		0/3	0/9	0/9	1/9	0/9	2/9	0/9	1/5			
P. undecaurelia	0/2	0/2	0/2			0/2	0/2	0/2	0/2	0/2	0/2	0/1			
P. dodecaurelia	0/3	0/3	0/3		1/3	0/1	0/1	0/1	0/1	0/1	0/1	1/5			
P. tredecaurelia	0/3	0/3	0/3		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3			
Total	2/141	15/227	8/209	1/27	14/204	0/245	0/245	3/245	1/245	3/245	0/245	12/97	2/11	1/8	1/7
%	1.4	6.6	3.8	3.7	6.9	0	0	1.2	0.4	1.2	0	12.4	18.2	12.5	14.3

[a] *P. decaurelia* and *P. quadecaurelia* are not included since each of these species has only one stock.

[b] Key to abbreviations: EstA = Esterase A, EstB = Esterase B, $EstC_C$ = Cathodal C esterase, $EstC_A$ = Anodal C esterase, Acp = Acid phosphatase, Fum = Fumarase, GDH = Glutamate dehydrogenase, HBD = β-hydroxybutyrate dehydrogenase, IDH_S = Isocitrate dehydrogenase (supernate), IDH_M = Isocitrate dehydrogenase (mitochondrial), SDH = Succinic dehydrogenase, GPI = Glucosephosphate isomerase, MDH = Malic dehydrogenase, mtDNA = Mitochondrial DNA, rDNA = Ribosomal DNA; and numbers in parentheses under abbreviations = references to the literature.

sett Pond in England (6). It is difficult to explain the presence of variability in *P. biaurelia* and its absence in other species for the same enzymes by the action of selection.

Differences in the ecology of the species are not well characterized. Distribution patterns suggest different temperature optima (31) and preferences for running or standing water (18). *P. biaurelia* is often found living sympatrically with other species of the *aurelia* complex in apparently identical environments; however, Gill & Hairston (17) found that it was particularly successful in competition with *P. pentaurelia.* They speculated that *P. biaurelia* may be particularly well adapted for colonizing rare islands of favorable habitat in coarse-grained environments. This might possibly explain its propensity for greater diversity in several biological parameters (31, 32) and its greater variability for certain enzymes.

One of the more unusual aspects of our data is the virtual absence of geographical differentiation of stocks within a species. This was noted previously (1). What is new is that it applies not only to the common ("wild-type") alleles but also to the variant alleles. Moreover, the larger number of stocks includes an even wider range of geographical locales. We would expect that populations so far apart must be completely isolated genetically. Lack of geographical differentiation cannot be explained by the absence of intraspecies variation because variants *are* found in these species, but in extremely low frequency.

These results are unexpected because cytogenetic studies have suggested local diversification of stocks and barriers to gene flow. The micronuclear chromosome numbers vary in different stocks of *P. primaurelia* and *P. tetraurelia* and differences in chromosome number were found to be correlated with low fertility in crosses (14, 20). Crosses between stocks from the same populations were found to be highly fertile, while those between stocks from different populations and with different chromosome numbers were much less viable. A direct correlation was found in one study in which the greater the departure in chromosome numbers the less the viability of the F_2 (20). Such observations would predict that stocks in the same species but from different parts of the world would accumulate different mutations and these would be expressed because of homozygosity resulting from autogamy. We would expect as a result of genetic drift or natural selection operating in different ways on these populations, that they would diverge and become highly polymorphic.

Considerable local differentiation of the populations would, therefore, have been expected for the enzymes. The lack of geographical differentiation suggests that strong selective forces are acting to maintain the "wild-type" alleles in high frequencies and the variant alleles in low frequencies within a species. Lack of geographical differentiation is also a feature of the distribution of endosymbionts and is found for the rDNA spacer sequences and has been used to argue for an ancient evolutionary origin for these species (15, 27). If these species did evolve some 100 million years ago, as suggested, then the enzyme allele established in a particular species must be under extremely stringent selection so that it is maintained. A variant allele must arise by recurrent mutation, although at low frequency, since it appears in widely separated populations. The observation that enzyme variations are clustered and that the frequency of intraspecies variation may be higher in certain parts of the world suggests that there are environments in which variant alleles can be more easily established.

Genetic polymorphism in the species complex. The results of electrophoretic studies can be used to estimate quantitatively the genetic variability in a species. One assumption made is that electrophoretic variation is the result of allelic substitutions at structural loci. Where genetic analysis has been carried out, this assumption is usually borne out. Genetic analysis has been carried out for the A esterases in *P. biaurelia* (7) and *P. octaurelia* (13), for the B esterases in *P. primaurelia* (13), *P. biaurelia* (7), and *P. tetraurelia* (8), for the cathodal C esterases in *P. primaurelia* (13), for glucosephosphate isomerase in *P. primaurelia* (37), for NADP-dependent isocitrate dehydrogenase in *P. biaurelia* (35), for NAD-dependent malic dehydrogenase in *P. tetraurelia* (38), and for β-hydroxybutyrate dehydrogenase in *P.*

novaurelia (34). For all of the enzymes examined above, the difference between the common and variant electrophoretic forms has been shown to be due to allelic differences at single loci.

How much genetic variation occurs in the *aurelia* complex as a whole? How does it compare to what is known for other organisms? Table VIII includes information on the frequency of variant stocks for 13 enzymes, mtDNA, and rDNA. For three enzymes (Fum, GDH, SDH) no variants have been observed in any of the species. For four enzymes (EstA, HBD, IDH_S, IDH_M) less than 2% of the stocks were observed to vary in the species complex as a whole, excluding *P. biaurelia* for EstA. For four other enzymes (EstB, $EstC_C$, $EstC_A$, Acp) the level of variation is 3–7% in the species complex, excluding *P. biaurelia* for $EstC_A$. For GPI (surveyed in all species), mtDNA (in two species), and MDH and rDNA (surveyed only in *P. tetraurelia*), the frequency of variation is higher (12–18%). In general, mtDNA and soluble enzymes show a high level of genetic variation in a wide variety of organisms compared to structural proteins (reviewed in 19). However, often there is no obvious association between the extent of polymorphism and an enzyme's molecular weight or biochemical function.

The amount of genetic variation observed for enzyme loci depends on the electrophoretic procedures used (19). Sequential electrophoresis seems to detect most amino acid substitutions, while one-dimensional electrophoresis, used in this study, detects only a fraction of the amino acid substitutions. Using one-dimensional electrophoresis to sample loci coding for soluble enzymes, the general rule for the many organisms surveyed is that ca. 28% of the loci show genetic variation, with most polymorphic loci having few alleles (26). This percentage was derived from taking the value for the overall mean heterozygosity for all organisms included in the analysis, 9.3%, and multiplying by a factor of three to compensate for underestimating total heterozygosity (26). Assuming that each enzyme in Table VIII is coded for by a single gene, and using the first 12 enzymes, the mean frequency of variation per locus is 3.1%, which when multiplied by three gives a value of 9.3%. Thus, a mean of 9.3% of the loci in the *aurelia* complex can be estimated to be polymorphic. The four species examined in this paper have been sampled more extensively than other species in the complex; however, all four have percentages of polymorphic loci that cluster around the value for the complex as a whole. We conclude that the amount of genetic variation in the species of the *aurelia* complex is much less than that observed for other organisms. For the species more intensely studied here the level of genetic polymorphism is also much lower, although *P. biaurelia* shows much greater variability for two of the enzymes. In the next paper in this series (2), interspecies relationships in the *P. aurelia* complex will be updated and genetic distances assessed.

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Interspecies Relationships in the *Paramecium aurelia* Complex: Acid Phosphatase Variation[1]

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ABSTRACT. Up to five zones of acid phosphatase activity appear in gels after electrophoresis of detergent-treated extracts from 13 of the 14 species of the *Paramecium aurelia* complex. The overall pattern is somewhat similar for all species; differences in intensity and mobility of individual zones permit the grouping of these sibling species into eight groups. All 14 species can be identified using the procedure of enzyme electrophoresis, although two of them are more similar than is usually the case. Problems of misclassification are discussed in terms of the nature and frequency of variants. With the judicious choice of enzymes used to screen new stocks, these problems can be circumvented. Species relationships are updated using 11 enzymes. A dendrogram constructed from the matrix of genetic distances shows four clusters of species: (i) *P. biaurelia, P. triaurelia*; (ii) *P. primaurelia, P. pentaurelia, P. sexaurelia, P. novaurelia*; (iii) *P. septaurelia, P. undecaurelia, P. tredecaurelia, P. quadecaurelia*; and (iv) *P. tetraurelia, P. octaurelia, P. decaurelia, P. dodecaurelia*. Distances between the species are large, on the order of the differences between *Drosophila* species. The species are characterized by an extraordinary lack of geographical differentiation and great morphological similarity, which contrasts strongly with the molecular differentiation.

ASSESSING biological relationships—among organisms that show few morphological differences—by use of morphological criteria alone may lead to gross underestimation of their true evolutionary distance. This problem is particularly severe in microorganisms including the ciliated protozoa, which, in addition, lack a fossil record.

Originally, the taxon "*Paramecium aurelia*" was used to lump together a number of organisms that were morphologically similar but between which gene flow did not always occur. As more information became available, particularly through application of biochemical techniques, classification of this group of organisms achieved finer resolution. At first, the 14 subgroups were called "varieties" on the basis of specificity of the mating types, that is, the specific selectivity of the breeding relations (see 30, and references therein). Subsequently the 14 varieties were designated "biological species," or "syngens," but not given species status, on the grounds that mating type identification was difficult to learn and apply to new strains and that it was undesirable to base identification on living material that had to be maintained in the laboratory and could easily be lost or mixed up (30). When it became possible to freeze and store paramecia in liquid nitrogen efficiently, and the syngens could be identified by simple biochemical procedures (such as starch gel electrophoresis of enzymes), species status was at last given to the 14 syngens (31, 32), now considered sibling species of an "*aurelia* complex."

Previous work on intra- and interspecies enzyme variation in this complex has enabled all 14 species to be distinguished on the basis of phenotypic differences observed for nine enzymes (4–6, 34, 36). Seven out of nine of these enzymes show intraspecific variation that is controlled by alleles at seven loci, EstA, EstB, $EstC_C$, HBD, IDH_S, IDH_M, GPI (7, 8, 19, 33, 35, 36). Intraspecific variation has also been observed for three other enzymes, anodal esterase C ($EstC_A$), acid phosphatase (Acp), and malic dehydrogenase (MDH), but genetic studies have been carried out only for MDH (14, 37). $EstC_A$ is not found in all species, and MDH has been examined only in *P. tetraurelia*. Acp is found in all species examined and is the subject of this paper. For some enzymes the variant forms are unique and do not coincide in mobility with those observed in interspecies comparisons (34). For the esterases the majority of the variants in *P. primaurelia, P. tetraurelia,* and *P. octaurelia* possess an electrophoretic mobility characteristic of a subtype observed in another species, whereas the mobilities of most of the variants in *P. biaurelia* are unique, as are the common forms in this species (8). About half of the variant forms for GPI coincide with forms found in other species (36). The frequency of intraspecific variation differs among enzymes (see Table VIII in Ref. 14). Less than 2% of the stocks were variant for seven enzymes, and 3–7% were variant for four other enzymes in the species complex as a whole, with *P. biaurelia* showing hypervariability for EstA and $EstC_A$. The frequency of variation was higher for GPI, 12.4% (36), and similar to that observed for MDH, mtDNA (mitochondrial DNA), and rDNA (ribosomal DNA) in *P. tetraurelia* (24, 25, 37).

Interspecies comparisons at nine enzyme loci led to estimation of genetic distances between the species of the *aurelia* complex (1). Not only were the differences between species large, but dendrograms constructed from the matrix of genetic distances indicated a more complex set of relationships between the species than that originally proposed by Sonneborn. Comparison of the genetic distances for this complex with similar values obtained for the *Tetrahymena pyriformis* complex and *Drosophila* sibling and nonsibling species led us to strongly urge species status for members of the *P. aurelia* complex.

Electrophoretic resolution of the acid phosphatases in the *P.*

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