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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.*

TABLE I. List of stocks among species of the Paramecium aurelia complex.

Species	Stocks	Variant for Acp	Geographical origin
P. primaurelia	44 stocks tested	60, 244, 258, 540, 561	See Table I in Ref. 14 for details on individual stocks
P. biaurelia	86 stocks tested	88, 185, 187, 560, 1010, 1050	See Table II in Ref. 14 for details on individual stocks
P. triaurelia	13, 70, 152, 275	—	U.S.A. (Maryland, Indiana, Connecticut, Alaska)
P. tetraurelia	27 stocks tested	278	See Table III in Ref. 14 for details on individual stocks
P. pentaurelia	76, 87, 311	—	U.S.A. (New Jersey, Pennsylvania), Australia
P. sexaurelia	309	—	Thailand
P. septaurelia	38, 227, 253	—	U.S.A. (Florida)
P. octaurelia	27 stocks tested	131	See Table IV in Ref. 14 for details on individual stocks
P. novaurelia	204, 317, 338	—	Scotland, France, U.S.S.R.
P. decaurelia	223	—	U.S.A. (Florida)
P. dodecaurelia	271, 273, 274	273	U.S.A. (Florida, Louisiana)
P. tredecaurelia	209, 238, 321	—	France, Madagascar, Mexico
P. quadecaurelia	328	—	Australia

aurelia complex has only recently been accomplished, since this enzyme is very tightly bound to membranes in Paramecium (14). This enzyme has now been examined in stocks of all the species complex except for P. undecaurelia. Data on this enzyme can now be added to the accumulated data on other enzymes to assess species relationships and update genetic distances. In a subsequent communication, we will consider other species complexes in Paramecium (P. multimicronucleatum, P. jenningsi, and P. caudatum) and compare the esterases and acid phosphatases in these species complexes with those of the aurelia complex (15).

MATERIALS AND METHODS

Stocks. The stocks listed in Table I were obtained from the Sonneborn Collection at Indiana University, now housed at the American Type Culture Collection. Cultures of this collection growing on living bacteria were kindly sent by Myrtle V. Schneller. They were transferred to axenic culture by methods described elsewhere (5, 12). 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 are described in Allen & Nerad (12).

Growth of the cultures for extracts. 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 at 23°C (13).

Identification of acid phosphatases. Extracts of cell concentrates harvested by centrifugation were frozen-thawed before extraction with the detergents Triton X-100 and sodium deoxycholate (14). Electrophoresis was carried out in 12% starch gels using Electrostarch and a Tris-boric acid buffer, pH 7.7, containing sodium deoxycholate, to which Triton X-100 and a few drops of antifoam were added after cooking and before degassing (14). Conditions for running the gels and cutting and staining them after electrophoresis have been previously described (10, 14). The reaction mixture for visualizing the enzyme 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 incubating the gel strips for 30–45 min at 23°C, they were rinsed with distilled water,

covered with 7% (v/v) acetic acid, and refrigerated until photographed.

RESULTS

Acid phosphatases. Simple freeze-thawing releases acid phosphatases from most of the species in the Tetrahymena pyriformis complex (16) and from Paramecium multimicronucleatum, syngen 3. Several bands appear on the gels in the case of the Tetrahymena species, and a couple of faint bands are seen for P. multimicronucleatum, syngen 3. Although some enzyme staining of extracts made from stocks of several of the species of the aurelia complex occurred, no bands appeared on the gels, even after varying buffer systems, pH, adding various ions, etc., nor did sonication of the extracts cause solubilization sufficient to resolve on gels. Several different detergents were tried (Brj58, Triton X-100, Nonidet P40, sodium deoxycholate), and all except Brj58 resulted in release of enzyme. Most effective was Triton X-100, which caused a fourfold increase in enzyme rate, and a sevenfold higher saturation level of the enzyme compared to control extracts of Paramecium. A more modest enhancement of activity was seen for Triton X-100 treated Tetrahymena extracts. Despite the increased release of enzyme, only smears were observed in the gels after electrophoresis of Triton X-100 treated Paramecium extracts. Dialysis, or centrifugation, of the extracts did not improve the resolution in the gel. However, when detergents were added to the gel, resolution of discrete enzyme forms was observed. This behavior 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. When Triton is added to the gel buffer, the micelle structure is maintained and resolution and migration improve dramatically. The addition of both Triton and sodium deoxycholate in the gel buffer results in still greater mobility and resolution.

Up to five zones of activity appear in the gels after electrophoresis of detergent-treated extracts from 13 of the 14 species of the aurelia complex. These are labeled A, B, C, D, E in order of increasing mobility in the diagrams (Fig. 1). The overall pat-
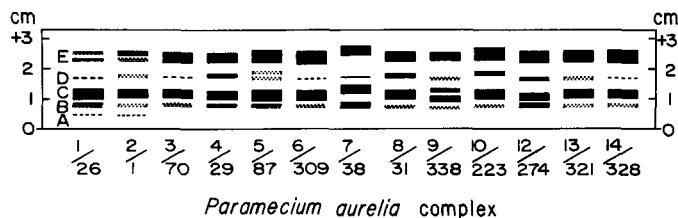
Fig. 1. Comparison of the acid phosphatases in members of the *Paramecium aurelia* complex. Species and stocks are designated by number/number (e.g., 1/26 refers to *P. primaurelia*, stock 26; 2/1 to *P. biaurelia*, stock 1; etc.). The relative intensities of the bands are indicated by the intensity 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 of the diagram.

TABLE II. *Distribution of enzyme subtypes among species of the* Paramecium aurelia *complex.*[a]

Enzyme	Subtypes (cathodal → anodal)
EstA	(9) (10) (1, 3, 5, 7, 11, 13, 14) (6, 12) (2) (4, 8)
EstB	(2) (1, 3, 5) (6) (9) (7, 13) (10, 14) (12) (11) (8) (4)
EstC$_C$	(2, 8) (6) (4) (14) (1, 3, 5, 9, 10, 12) (7, 11, 13)
Fum	(4, 8, 10, 12, 13) (2, 3, 7) (1, 5, 6, 9, 11, 14)
GDH	(10, 11) (1, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14) (2)
HBD	(6, 8) (7, 14) (1, 5, 10, 12) (2, 3) (4, 9, 11) (13)
IDH$_S$	(12) (13) (10) (11) (2, 3) (1, 5, 6, 7, 8, 9, 14) (4)
IDH$_M$	(6) (4, 10, 12) (13) (1, 2, 3, 5, 7, 8, 9, 11, 14)
SDH	(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14)
GPI	(12) (2) (1, 7)[b] (4, 8, 10) (11, 13, 14) (3) (5) (6) (9)
Acp	(9) (1, 2, 3) (5) (4, 8) (6, 13, 14) (12) (10) (7)

[a] Key to abbreviations: Numbers refer to species (e.g., 1 = *P. primaurelia*, 2 = *P. biaurelia*, etc.). Enzymes: EstA = Esterase A; EstB = Esterase B; EstC$_C$ = Esterase C$_C$; 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; Acp = Acid phosphatase.

[b] Genetic identity (I; see Ref. 28) between species 1 and 7 is 0.70 for this enzyme. For all other comparisons of enzymes, I is ≈0.0 or ≈1.0.

tern is somewhat similar for all species. However, there are differences in intensity of individual zones, and in mobility, within the pattern as a unit when different species are compared. The patterns observed for *P. primaurelia*, *P. biaurelia*, and *P. triaurelia* appear to be similar except for minor differences in the intensity of individual isozymes in the doublet found in zone E. *P. pentaurelia* has this doublet, but it differs from the other species in having a second doublet in zone D. The patterns observed for *P. tetraurelia* and *P. octaurelia* appear similar but differ slightly from those found in *P. sexaurelia*, *P. tredecaurelia*, and *P. quadecaurelia*. The remaining species, *P. septaurelia*, *P. novaurelia*, *P. decaurelia*, and *P. dodecaurelia*, each appear to have a unique pattern. Variant stocks were found in *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. octaurelia*, where a large number of stocks was screened (Table I). Only one stock exists for *P. decaurelia* and *P. quadecaurelia*, and only one stock was tested for *P. sexaurelia*. Three stocks each were examined in *P. pentaurelia*, *P. septaurelia*, *P. novaurelia*, *P. dodecaurelia*, and *P. tredecaurelia*, and four stocks in *P. triaurelia*, and no variants were observed in these six species, except for stock 273 in *P. dodecaurelia*. For eight of the 10 species where more than one stock was tested, most of the stocks were collected from widely separated geographical areas (14; and see our Table I). The patterns of the variants differed in intensity, or in mobility of one or more of the five zones that make up the pattern, when compared to the commonly observed pattern.

Since there are problems in the resolution of the enzyme forms in *Paramecium*, and in their limited mobility, the pattern of bands has been treated as a unit. By contrast, some 18–20 well resolved bands with a much greater spread in their mobility appear after simple freeze-thawing of extracts from *T. thermophila*. Moreover, individual bands in this species differ in their activities with different substrates and inhibitors, in their sensitivity to neutral and alkaline pH, and in their genetic control (2, 3, 10, 11). Thus, in *Tetrahymena* a family of enzymes is seen, with different gene products being displayed. The situation in *Paramecium* may also be more complicated; however, until better resolution can be achieved, we prefer to treat the pattern we see as a unit.

Updating species relationships. The results with the acid phosphatases allow us to distinguish eight groups of species in the *P. aurelia* complex: *P. novaurelia*; *P. primaurelia*, *P. biaurelia*, *P. triaurelia*; *P. pentaurelia*; *P. tetraurelia*, *P. octaurelia*; *P. sexaurelia*, *P. tredecaurelia*, *P. quadecaurelia*; *P. dodecaurelia*; *P. decaurelia*; and *P. septaurelia*. Importantly, this gives a second enzyme that allows discrimination between *P. primaurelia* and *P. pentaurelia*. Species that cannot be distinguished using Acp can be distinguished with other enzymes, making it possible to identify all 14 of the known species of the *P. aurelia* complex.

Table II shows the differences between species of the *aurelia* complex for 11 enzymes. In compiling the groupings in this table we have used the most common enzyme subtype for each species. For most enzymes and species the level of intraspecific variability is low and interferes little in making the groups. For EstA, there are 12 subtypes in *P. biaurelia* but only one is similar in phenotype to another species and was found in only two out of the 86 stocks surveyed (8). A high level of intraspecific variation exists for GPI, and in one case, comparison between *P. primaurelia* and *P. septaurelia*, no clear grouping exists.

Table III shows the pairwise genetic distances calculated for all species taking into account both interspecific variation and intraspecific variation. In general, the table shows that the distances between species are large, with values ranging from a low of 0.431 for *P. primaurelia* and *P. pentaurelia* to a high of 0.954 for several of the comparisons. The mean euclidean distance between all species is 0.825, slightly higher than the value obtained previously (1).

Figure 2 shows the dendrogram calculated from the matrix of genetic distances. This represents the best estimate of the minimum length tree describing the relationships between the species. This tree was derived using the algorithm of Cavalli-Sforza & Edwards (20) starting from an initial cluster obtained using the divisive algorithm of the same two workers (23). The same considerations, as discussed earlier (1), apply in interpreting this tree as a representation of the evolution of this group of species. The dendrogram shows that there are four clusters of species. *P. tetraurelia*, *P. octaurelia*, *P. decaurelia*, and *P. dodecaurelia* are grouped together; *P. septaurelia*, *P. undecaurelia*, *P. tredecaurelia*, and *P. quadecaurelia* group together; *P. biaurelia* and *P. triaurelia* are closely related; and *P. primaurelia*, *P. pentaurelia*, *P. sexaurelia*, and *P. novaurelia* form a cluster. The earlier dendrogram (1) also showed four clusters; however, three differences should be noted: *P. primaurelia* is now separated from *P. pentaurelia*; *P. octaurelia* is now grouped with *P. tetraurelia*; and *P. quadecaurelia* is now grouped with *P. septaurelia*. The groupings in the present dendrogram are reinforced by data on the similarity of variants to common forms or variants in other species, especially for *P. tetraurelia* and *P. octaurelia* (8). They also support, in part, Sonneborn's (30) conclusion that *P. biaurelia*, *P. tetraurelia*, and *P. octaurelia*
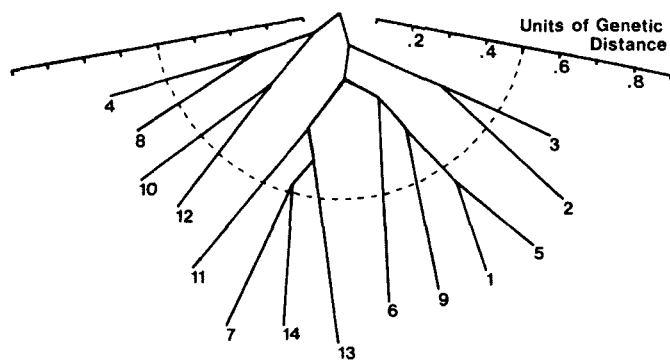
Fig. 2. Dendrogram of the 14 species of the *Paramecium aurelia* complex. The dendrogram represents the "best" topology based on the criterion of minimum evolution. The networks are drawn to scale and plotted on polar coordinates so that distances between populations are read off along the radii. The tree is unrooted; however, the dendrogram is drawn such that the segment joining the results of the primary split passes through the origin.

formed one group and *P. primaurelia, P. triaurelia,* and *P. novaurelia* formed another group on the basis of morphological and antigenic differences. Analysis of the isozyme data places *P. biaurelia* and *P. triaurelia* together and suggests a more complex pattern of relationships for the species complex as a whole.

DISCUSSION

Species classification. Although all 14 species can be identified using the procedures of enzyme electrophoresis, without due care stocks of some species may easily be misclassified. For example, *P. primaurelia* and *P. pentaurelia* differ for only two of the 11 enzymes assayed, and, without recourse to information on these enzymes, stocks of these two species may not be distinguished. The existence of enzyme variants in one species that are similar to the common forms or variants of other species also creates the potential for misclassification. For most enzymes the level of intraspecific variation is extremely low, so this problem is minimal. However, in four species—*P. primaurelia, P. biaurelia, P. tetraurelia,* and *P. octaurelia*—the variations are clustered in several of the variant stocks (14). Such clustered variation exacerbates the problem of misclassification. With judicious choice of enzymes, an unidentified stock may

be assigned to one of the 14 species without danger of misclassification. We suggest the use of four key enzymes, EstA, EstB, GPI, and IDH (or Fum or HBD), taking into account the pattern of crossreaction between species, of variants and common forms, to maximize the probability of a "one-shot" assignment. For any stock tentatively assigned to *P. pentaurelia,* additional enzyme tests (Acp) or mating type tests should be carried out.

Genetic distances between species. The 14 species may be distinguished with many less than the 11 enzymes used in our analysis. The distances between species are therefore quite large. The magnitude of the distances is reflected in the structure of the dendrogram (Fig. 2), where the lengths of all the terminal branches are long in relation to the size of the dendrogram itself. However, it is difficult to interpret the magnitude of these distances without reference to other groups of organisms. The mean genetic distance between species is 0.825. This value is somewhat higher than the value given for *Drosophila* sibling species but is lower than the figure for *Drosophila* nonsibling species (27) or for the *Tetrahymena* species (1). A similar pattern of results was found in comparisons of nucleotide sequence divergence between *Drosophila* sibling and nonsibling species and *Paramecium* and *Tetrahymena* species (9).

Nei (27) has estimated the time of divergence for *Drosophila* species to be between 10^5 and 10^6 years, and the same level of genetic distances for the 14 species of the *P. aurelia* complex suggests a similar time of divergence. However, such comparisons assume parsimony of evolution as well as equality in the rate of gene substitution per year across different genes and species. If other factors enter in to modify this rate, then estimates of genetic distances in different groups of species may not be equivalent. From the distribution of endosymbiont-bearing paramecia, Preer (29) argues for a much more ancient history, suggesting that present stocks have been geographically and sexually isolated since the early Cenozoic–late Mesozoic, or approximately 100×10^6 years ago.

The nature of the molecular data, the magnitude of the genetic distances, and the absence of a fossil record do not allow us to critically evaluate these two estimates of divergence time. Whatever this time may be, the molecular differentiation between the 14 species of the *aurelia* complex contrasts strongly with their lack of morphological differentiation (30–32). A similar contrast is seen in the species of the *T. pyriformis* complex. The morphology of all these species is very similar, in spite of the fact that from molecular data they display little or no similarity (17).

TABLE III. *Pairwise distance matrix for the 14 species of the* Paramecium aurelia *complex.*[a]

	1	2	3	4	5	6	7	8	9	10	11[b]	12	13	14
1	—													
2	0.852	—												
3	0.594	0.675	—											
4	0.905	0.954	0.905	—										
5	0.431	0.905	0.674	0.905	—									
6	0.791	0.954	0.905	0.905	0.787	—								
7	0.695	0.853	0.739	0.905	0.739	0.853	—							
8	0.792	0.860	0.853	0.671	0.800	0.800	0.800	—						
9	0.680	0.909	0.802	0.863	0.678	0.800	0.802	0.802	—					
10	0.856	0.954	0.905	0.800	0.853	0.954	0.954	0.856	0.905	—				
11[b]	0.767	0.895	0.837	0.902	0.775	0.894	0.775	0.894	0.784	0.894	—			
12	0.801	0.954	0.853	0.800	0.800	0.853	0.905	0.848	0.853	0.739	0.949	—		
13	0.846	0.954	0.853	0.853	0.853	0.853	0.739	0.853	0.905	0.905	0.775	0.853	—	
14	0.668	0.905	0.800	0.905	0.674	0.739	0.674	0.800	0.743	0.905	0.707	0.905	0.739	—
Species	1	2	3	4	5	6	7	8	9	10	11[b]	12	13	14

[a] Distance measures (d) are calculated using the relationship $d = \sqrt{1 - I}$. I is the measure of genetic identity of Nei (28) calculated using the data for the enzyme loci described in the text. Species 1 = *P. primaurelia,* species 2 = *P. biaurelia,* etc.

[b] Acid phosphatase not assayed for *P. undecaurelia.*

Nanney (26) suggests that substitutions that affect form/function relationships may be much more strongly selected against than other categories of substitutions, and that this may explain the apparent discrepancy between the morphological and molecular data. Certainly the assumption of differing rates of evolution for different categories of genes is not new (21). Recently, Brown and his co-workers (18) have provided evidence that the mitochondrial genome of higher vertebrates may have evolved much faster than the nuclear genome. In this regard it is interesting to note that the restriction endonuclease digestion pattern of mtDNA differed in one out of four stocks examined in *P. tetraurelia* (25).

Our results illustrate a further unique feature of the *aurelia* complex: namely, the extraordinary lack of geographical differentiation, in spite of the cosmopolitan distribution of some species, most notably, *P. primaurelia, P. biaurelia,* and *P. tetraurelia* (although the majority of stocks were collected from the United States). Lack of geographical differentiation is also a feature of the distribution of endosymbionts and is also found for rDNA spacer sequences. The lack of geographical differentiation for rDNA spacer sequences is especially surprising, given the large amount of natural variation found for such sequences in other organisms (e.g., *Drosophila*; see 22). Explanation for this level of geographical uniformity must await future studies on the genetics, evolution, and ecology of this species complex.

In the next paper, we turn to other species complexes in *Paramecium*. We will compare the esterases and acid phosphatases in the five syngens of *P. multimicronucleatum, P. jenningsi,* and *P. caudatum* with those of the *aurelia* complex (15).

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