directly, and that this protection is not provided when calcium is replaced by strontium.

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Distribution of *Tetrahymena pyriformis* in Europe*

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SYNOPSIS. One hundred eighty-eight fresh-water samples from France (30), Italy (42), Austria (12), Germany (1), Switzerland (35), Holland (20), England (26), and Sweden (22) were examined for *T. pyriformis.* The habitats included rivers, mountain streams, lakes, ponds, irrigation ditches, roadside ditches, running and dead water canals, and fountains. The temperature ranged from 10° to 30°C, locations from 42° to 68° North latitude, with elevations from sea level to approximately 3,000 feet. Of the samples taken 28 contained *T. pyriformis* from which 411 clones were established in axenic media. Thirty-three additional samples contained ciliates other than *T. pyriformis.* All clones grew well in 1% proteose peptone; when screened for their nutritional requirements a few failed to grow on the completely defined medium and several others survived eight sub-inoculations without thiamine.

The nuclear and sexual activity pattern followed that of previous collections. In distribution, variety 6 was found in Italy, variety 3 in Austria, and variety 4 in England. A new variety (variety 10) with two mating types was isolated from four different habitats in England. Several strains from Italy, France, Holland, and England constitute one group which mated among themselves, but only a few of which reacted with mating type I of variety 6 from America. This demonstrates the close affinities of the European and American strains, yet shows the possible evolution of a new variety (species).

*T*etrahymena pyriformis is widely distributed in the Western Hemisphere as revealed by a number of investigators(1,11,13). It has been found in Europe on several occasions; indeed, the first axenic culture of this organism was reported by Lwoff(17), although his strain was then known as *Glaucoma pyriformis.* More recently it has been identified as *T. pyriformis,* strain GL(4). Later Chatton(3) isolated another strain now known as *T. pyriformis* strain CH-S. Robertson(16) reported *Glaucoma pyriformis* in England which has also been identified as *T. pyriformis*(4). *T. pyriformis* is widely distributed over the Americas. Intensive studies have shown it to be generally distributed over the United States(11,13). Its appearance in collections taken from Mexico, Panama, and Colombia indicated its probable distribution in Mid-Central and South America(12). It is highly

likely that *T. pyriformis* occurs on most of the land masses of the world.

It is now known that all of the earlier strains are sexually inactive, amicronucleate strains, hence cannot be classified as to variety and mating type(4). This fact precludes a study of the distribution of the species based on genetic affinities. Whereas morphological detail is reasonably adequate to identify the species, cross-breeding experiments are the only criteria that can be relied upon to delineate the varieties. If one wishes to study the evolution of this species it becomes necessary to examine strains taken from diverse habitats and from as many areas of the earth as possible. These must then be relegated to varieties by cross-breeding experiments. Once this information is assembled, varietal affinities can be determined by hybridization experiments including testing for progeny viability. A rather complete picture of the geographical distribution in correlation with land masses should emerge, from which some understanding of the

evolution of this protozoan species may become clear.

To this end *T. pyriformis* was collected from England and Western Europe during the summer of 1958. These clones supplement our earlier collections(11,12, 13). The purpose of this report is to describe the outcome of this venture.

MATERIALS AND METHODS

The methods of collecting and caring for the samples were essentially the same as outlined in a previous report(12). One variation was introduced which has become standard laboratory procedure. The cerophyll medium is dispensed with entirely. *Aerobacter aerogenes* is grown in nutrient agar in 500 ml flasks for 24 hours to which 500 ml double glass-distilled water is then added. By shaking, the bacteria are evenly suspended, adjusted to pH 6.8, and then pipetted to tubes for use. The ciliates grow well on this medium and consume the bacteria quickly. Stock cultures are maintained in this medium until they are rendered axenic, at which time they are grown in the following medium:

Bacto-Tryptone	5.0	g/liter
Proteose peptone	5.0	g/liter
Sodium acetate	1.0	g/liter
KH$_2$PO$_4$	1.0	g/liter
Liver extract	0.1	g/liter
Thiamine HCl	0.002	g/liter

The ciliates were cloned after arrival in Ann Arbor, then subjected to antibiotics according to a procedure described earlier(12) in order to establish axenic lines. They were then tested for variety and mating type in the following manner.

The clones were grown to maximal levels in the stock medium (36 to 72 hours), washed three times in glass-distilled water, and then allowed to starve from two to 12 hours. Cells that did not survive this treatment were discarded and only active organisms were used in the mating tests. Frequently, when clones are mixed, pairs form but are loosely attached and soon separate without undergoing meiosis and cross-fertilization. These are obviously not opposite mating types. Only when a number of firmly attached pairs appeared in the depression was the reaction recorded as positive. Moreover, whenever there was some doubt as to the reaction the cross was repeated until the results were conclusive.

All clones were treated with a lacto-orcein-gelatin staining schedule to determine the presence or absence of a micronucleus. This method is reliable only when the micronucleus is normal in size; hence those clones that appeared amicronucleate with this technique when subjected to the Feulgen reaction revealed very small micronuclei in some cases. These may be haploid clones(7).

Owing to aging it has become necessary to constantly crossbreed our tester stocks in order to assure not only satisfactory mating, but also to maintain progeny viability, which constantly drops with time. Hence, most of the original tester stocks have now been replaced by progeny several generations removed from the parental lines. Our present testers, together with their history and varietal distribution, are listed below:

Variety One (United States): Mating types I (WH$_6$) and II (WH$_{14}$) were isolated from the vicinity of Woods Hole, Massachusetts, in 1952(10), from which seven mating types were derived by Nanney and Caughey(18) and constitute the present seven mating types of the testers.

Variety Two (United States, Mexico, and Panama): Ten mating types, originally isolated by Gruchy(13) and an 11th by Elliott and Hayes(12), were examined by Hurst(14) who discovered that there were only 9 valid mating types for which the original numbers have been retained. Mating types V and XI are now obsolete.

Variety Three (United States and Austria): Seven mating types were originally isolated by Gruchy(13); Byrd(2) in revitalizing the lines found no additional mating types.

Variety Four (United States and England): The three mating types found by Gruchy(13) are still being used although 4/III, which no longer conjugates, has been replaced by EN 131-18—a more vigorous strain.

Variety Five (United States): Gruchy's(13) original two mating types are still in use since no viable offspring have been derived.

Variety Six (United States and Italy): Gruchy's(13) original three mating types are still in use.

Variety Seven (United States): Outka(21) derived two additional mating types from the two found by Gruchy(13).

Variety Eight (United States): Orias(19,20) has derived viable offspring from the three original mating types(13), although no additional mating types have been found.

Variety Nine (Panama and Colombia): The original five mating types found in Central America by Elliott and Hayes (12) are still in use although work is now in progress to improve the viability of these strains.

Variety Ten (England): The two original mating types found during this study are now being crossed to improve viability.

Once the variety and mating type were determined each clone was tested for growth on the chemically defined medium that supports strain E(12). The cells were washed three times in sterile glass-distilled water and then allowed to starve for six hours before being inoculated into the defined medium. The customary omission technique was employed with periodic checks for contaminating bacteria whenever growth occurred on deficient media. Clones were tested in groups of 10 to 20 in order to facilitate the program. Only in those cases where growth occurred in media lacking a single nutrilite were the clones of that group checked individually. If the clone grew satisfactorily through seven transfers (0.08 ml inoculation) in deficient media, it was considered a mutant. Likewise, clones that maintained good growth in the complete defined media were labelled as wild types.

RESULTS—Areas Studied

One hundred eighty-eight fresh water samples from seven continental European countries and England were examined for *T. pyriformis*. The habitats included rivers, small streams, lakes, ponds, irrigation ditches, running and dead water canals, and fountains. The temperature ranged from 10° to 30°C, locations from 42° to 68° North latitude, with elevations from sea level to approximately 3,000 feet. The sample number, location of the habitat, strain number, presence or absence of a micronucleus, variety and mating type are given in Tables 1 and 2. The strain designation is the habitat number followed by a number which indicates the number of the clone isolated from the sample. This is preceded by EU for those clones taken from continental Europe and EN for those from England. This numbering system was adopted earlier (12).

Austria: Twelve samples (#59 to #70) were taken in Austria, most of which came from the River Inn and its tributaries. As in most cases the samples were obtained from habitats near the road, usually from streams intersecting the highway. In mountain passes, owing to the precipitous nature of the terrain, it was

TABLE 1. *Samples from continental Europe containing* T. pyriformis *collected during the summer of 1958. Each country is listed separately giving total samples and percentage positive.*

Sample number	Location	Strain number	Micro-nuclei	Variety	Mating type
AUSTRIA, 12 samples taken (#59-#70) 4 positive (33%)					
EU 61	River Inn, Innsbruch	61-2	0	—	—
EU 62	River Inn, Innsbruch	62-4	0	—	—
EU 64	Mountain stream, Arlberg	64-1	+	3	V
		64-2 thru 64-24	0	—	—
EU 64A	Mountain stream, Arlberg	64A-2, 9, & 11	0	—	—
EU 67	Ditch near Feldkirch	67-1 thru 67-24	0	—	—
FRANCE, 30 samples taken (#1-#16, #133-#146) 3 positive (10%)					
EU 9	Beaumont-s-Sarthe	9-1 thru 9-24	0	—	—
EU 10	Beaumont-s-Sarthe	10-1 thru 10-12	+	6 (European)	I
EU 143	Stream in park of Versailles Palace	143-1 thru 143-24	+	6 (European)	II
GERMANY, 1 sample taken (#71) negative for *T. pyriformis*					
HOLLAND, 20 samples taken (#147-#166) 3 positive (15%)					
EU 147	Amsterdam Canal (running water)	147-3, 8, & 11	+	6 (European)	I
EU 149	Amsterdam Canal (dead water)	149-1 thru 149-12	+	6 (European)	I
EU 149A	Amsterdam Canal (dead water)	149A-1 thru 149A-12	0	—	—
EU 166	Canal in Leiden	166-1 thru 166-10	+	Selfers	
ITALY, 42 samples taken (#17-#58) 5 positive (12%)					
EU 17	LaSpezia, Vara River tributary	17-1 thru 17-24	0	—	—
EU 30	Lake Bolsena	30-1 thru 30-24	0	—	—
EU 40	Stream near Bologna	40-1 thru 40-24	+	6 (European)	I
EU 49	Stream near Padua	49-1 thru 49-12	+	Selfers	
EU 56	Canal near Borgo	56-14	+	6 (American)	II
		56-1 thru 56-24	+	?	?
SWEDEN, 22 samples taken (#167-#188) 1 positive (5%)					
EU 188	Stream near Kiruna	188-1 thru 188-12	+	?	?
SWITZERLAND, 35 samples taken (#72-#106) 2 positive (6%)					
EU 86	Berne—pond behind Zool. Institute	86-1 thru 86-24	+	?	?
EU 104	Stream near Lausanne	104-5	+	Selfer	

necessary to obtain the sample by means of a container and a fishline. In all cases an effort was made to take the quiet water near the edge since very few ciliates have been found in the rapidly moving portions of a stream. Four of these habitats contained *T. pyriformis* of which only one clone, EU 64-1, was sexually active and turned out to be 3/V. This one was obtained from a small mountain stream near Arlberg. All other clones proved to be amicronucleate.

France: The collections taken in France were disappointing in that only 10% (3 out of 30 habitats) were positive for *T. pyriformis*. However, most of the habitats contained a species of *Glaucoma* which grew well on bacterized water but failed when rendered axenic. Collections were made in France on two different occasions. The first (#1 through #16) were made in June and included habitats located along highways from Cherbourg to Cannes. The streams of

this gentle rolling country seemed to be idyllic spots for the ciliates although they appeared in only two of the 16 habitats. Both of these were from different parts of the same river system (Sarthe). One (EU 9) yielded 24 amicronucleate clones while from the other (EU 10) 12 sexually active clones were isolated. These turned out to be European 6/I. The second series of collections (#133 to #146) were made in the vicinity of Paris (August 2nd through 5th) of which only one harbored *T. pyriformis*. From this sample (EU 143) 24 clones were established all of which turned out to be European 6/II. Throughout this paper American variety 6 refers to clones which react completely with our variety 6 testers; European variety 6 refers to clones which show only partial reactivity, the details of which will be dealt with in the discussion.

Germany: The one sample taken in Germany (#71)

TABLE 2. *Samples from England containing* T. pyriformis *collected during the summer of 1958. Twenty-six samples were taken (#107–#132) of which 10 were positive (39%).*

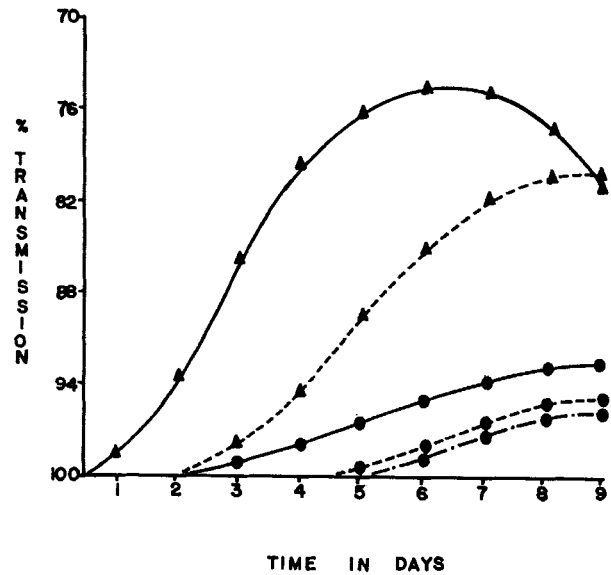| Sample number	Location	Strain number	Micro-nuclei	Variety	Mating type
EN 107	Lister Pond, London	107-1 thru 107-12	+	Selfers	
EN 112	Hunter Bridge Canal	112-3 & 10	+	10	I
		112-1 thru 112-24 (excluding 3 & 10)	+	?	?
EN 113	Stream near Apsley	113-1 thru 113-24	0	—	—
EN 117	Stream near Waddesdon	117-1 thru 117-12	0	—	—
EN 118	Stream near Bicester	118-1 thru 118-3 & 5	+	4	III
EN 120	Stream near Newton Purcelle	120-1 thru 120-12	0	—	—
		120-13 thru 120-24	+	6 (European)	II
EN 121	Stream near Avondale	121-1 thru 121-9	+	10	II
EN 130	Stream near Amersham	130-1 thru 130-13	+	10	II
		130-14 thru 130-24	+	?	?
EN 131	Stream near Amersham	131-1 thru 131-10	+	10	II
		131-11 thru 131-24	+	4	III
EN 132	Stream near Uxbridge	132-1 thru 132-24	+	4	III

TIME IN DAYS

Fig. 1. Growth curves for the temperate zone strain E (solid triangles) and the cold-adapted strain EU 188 (solid circles) are compared when grown at 4° (— • — •), 15° (— — — —), ar 25°C (———). Whereas strain EU 188 grows poorly at 15, and 25°C when compared to strain E, it grows moderately well at 4°C which kills strain E (no curve shown).

from Lake Constance contained no *T. pyriformis.*

Holland: Twenty samples were taken (#147 through #166) from rivers, lakes, and the largest number from canals, some of which contained impounded water that had essentially no circulation (dead canals) where many species of protozoans flourished. The three samples containing *T. pyriformis* came from canals, two in the vicinity of Amsterdam (EU 147 and EU 149) and one in Leiden (EU 166). Samples EU 149 and EU 149A were taken from the same habitat, the former yielded clones belonging to European 6/I, and from the latter amicronucleates were isolated. Clones from the Leiden canal (EU 166) turned out to be selfers, whereas clones belonging to European 6/I were isolated from another Amsterdam Canal (EU 147). These sexually active European 6/I clones will be considered later.

Italy: Water samples were taken along the coastal highway from Monaco to Rome, then north to Florence, Bologna, Padua, Venice, Bolzano, and over the Brenner Pass into Austria. The terrain was mountainous, the elevation varying from sea level to several thousand feet. Of the 42 samples taken only 5 yielded clones of *T. pyriformis,* two of which were amicronucleate (EU 17 and EU 30). One of the three micronucleate clones (EU 40) turned out to be European

6/I. The second group of micronucleate clones (EU 49) consisted of selfers and all but one of the third group (EU 56-1 through EU 56-13, EU 56-15 through EU 56-24) failed to mate with any of our testers so may belong to a new variety. The one clone (EU 56-14) of this group turned out to be American 6/II. The habitats of European 6/I (EU 40) and American 6/II (EU 56-14) were some 150 miles apart and from different river systems.

Sweden: The only collections made in Sweden were in the vicinity of Kiruna which is 68° North latitude, above the Arctic Circle in Lapland. It was hoped that if *T. pyriformis* could be found in these latitudes, some unusual physiological characteristics as well as breeding behavior patterns might be encountered when compared with ciliates from temperate and tropical regions. Twenty-two collections were taken from ponds and small streams within a radius of 25 miles of Kiruna. Of these only one contained *T. pyriformis* from which twelve clones were isolated. These possess a small micronucleus but do not mate with any of the testers, hence, probably belong to another variety. Since the micronucleus is unusually small, it may be haploid.

As one might expect these clones tolerate low temperatures, growing slowly at 4°C. No other clones we have tested survive this temperature. The growth rate of a representative clone at 4°C, 15°C, and 25° C is compared with strain E in Fig. 1. In spite of its cold tolerance it cannot survive freezing any better than strains found in warmer environments. Attempts

to freeze the cells in a variety of media, including 15% glycerol, as well as at different rates of freezing, resulted in death of the cells. A puzzling question is how this organism survives its environment which certainly is frozen a good part of the year, yet cannot survive laboratory freezing within the limits of our experiments.

Switzerland:[1] In spite of an ample number of samples (#72 through #106) taken from Rorschach in the northeast to Geneva in the southwest, the number containing *T. pyriformis* (2 samples) was disappointing, although *Glaucoma* appeared in many of the samples. It may only be an impression, but it seems that when this species occurs, *T. pyriformis* is absent. It is possible that competition between the two is sufficiently great to permit the predominance of only one in certain areas. Of the two micronucleate groups, one (EU 104-5) was a selfer, and the other (EU 86) refused to mate with any of the testers.

England: *T. pyriformis* appeared more frequently in samples taken in England than from the continent; indeed the highest percentage in this study (39%). Twenty-six collections were made along the highways 100 miles northwest of London and in the city itself of which 10 contained *T. pyriformis*. Small streams were the principle habitats although selfing clones were found in a small pond, "Lister Pond," through the courtesy of Dr. Muriel Robertson.

Of the ten habitats harboring *T. pyriformis*, three (EN 118, EN 131, EN 132) contained 4/III, one of which was separated from the other two by 25 to 30 miles. However, all three habitats were small streams making up a part of the Thames drainage system. One habitat (EN 120) contained European 6/II as well as amicronucleate clones. A new variety consisting of two mating types was found in four habitats, one of which (EN 131) also contained 4/III. This new variety designated variety 10, was found near London (EN 112, EN 130, EN 131) and again approximately 75 miles distant (EN 121), all habitats being a part of the Thames drainage system. It is noteworthy that both 4/III and 10/II were found in the same habitat (EN 131) which is the only case where two varieties have been found together in this entire study, although such association has been reported(12). In another habitat (EN 130) micronucleate clones were found that failed to mate with any of the testers. They could belong to an unknown variety or be senile, hence unable to mate. A similar situation existed in another habitat (EN 112). Amicronucleate clones appeared in three collections (EN 113, EN 117, EN 120) which, as expected, failed to mate. Unlike our earlier observations no habitat in England or on the Continent contained more than one mating type of any variety.

Variety 10—A New Variety: Morphologically these ciliates possess the characteristics that identify *T. pyriformis*. Log growth organisms possess about the same size range as strain E. The growth characteristics also resemble strain E, although both clones of 10/I (EN 112-3 and EN 112-10) grow somewhat slower than the 32 clones belonging to 10/II. Mating occurs readily reaching 100% when equal numbers are mixed. The refractory period is 4-5 hours at 25° C and the anlagen stage is reached in approximately 40 hours. There is no immaturity period.

The first crosses yielded 2.2% viability which increased to 12% in the F_2 generation. No further crosses have been attempted so far. It is likely that the viability will improve in subsequent generations.

Nutritional Analysis

Inasmuch as only sexually active ciliates were of interest for possible future genetic studies, no nutritional tests were undertaken with amicronucleate or non-mating micronucleate clones. The results of these tests indicated that many clones failed to survive in the chemically defined medium which supports strain E and/or the wild type, which meant that additional nutrilites were required. They were then subjected to a medium containing seven nutrilites in addition to those in the standard medium, the concentrations being those of Kidder and Dewey(15) with the exception of biotin whose concentration was greatly reduced. The composition of the medium follows with the additions marked by asterisks:

Amino acids:	mg/liter	Growth factors:	mg/liter
*Alanine	110	*Biotin	0.00025
Arginine	150	Ca pantothenate	0.10
*Aspartic acid	122	*Choline	1.0
*Glutamic acid	233	Folic acid	0.01
*Glycine	10	Niacin	0.10
Histidine	110	Pyridoxine HCl	2.00
Isoleucine	100	Riboflavin	0.10
Leucine	70	Thiamine HCl	1.00
Lysine	35	Thioctic acid	0.001
Methionine	35		
Phenylalanine	100	Carbon source:	
*Proline	250	Glucose	1000
Serine	180	Sodium acetate	1000
Threonine	180		
Tryptophane	40	Inorganic salts:	
Valine	60	K_2HPO_4	100
Nucleic acids:		$MgSO_4 \cdot 7H_2O$	10
Adenylic acid	25	$Zn(NO_3)_2 \cdot 6H_2O$	5
Cytidylic acid	25	$FeSO_4 \cdot 7H_2O$	0.5
Guanylic acid	25	$CuCl_2 \cdot 2H_2O$	0.5
Uracil	25		

The data for these tests are shown in Table 3. It will be observed that all of the clones belonging to variety 10 have the same requirements as the wild type with the exception of thiamine, which is not required. The thiamine requirement for the wild type (strain E) is obvious after 3 to 4 transfers whereas

TABLE 3. *Nutritional requirements of sexually active strains*

Collection number	Clones tested	Classification*	Habitat	Growth on chemically de-fined media (strain E)	Addt'l nutrilites	Requires thiamine
EU 10	12	6/I	Beaumont-s-Sarthe, France	+	0	+
EU 40	24	6/I	Bologna, Italy	—	7	+
EU 143	24	6/II	Versailles, France	—	?†	+
EU 147	3	6/I	Amsterdam, Holland	—	2	+
EU 149	12	6/I	Amsterdam, Holland	—	7	+
EN 112	2	10/I	Hunter Bridge Canal, England	+	0	—
EN 118	4	4/III	Bicester, England	—	7	+
EN 120	12	6/II	Newton Purcelle, England	—	4	+
EN 121	9	10/II	Avondale, England	+	0	—
EN 130	13	10/II	Amersham, England	+	0	—
EN 131	9	10/II	Amersham, England	+	0	—
	12	4/III	Amersham, England	—	7	+
EN 132	24	4/III	Uxbridge, England	—	7	+

* All clones listed as variety 6 belong to the European variety 6.
† Not tested for additional nutrilites.

these strains grow vigorously after 8 transfers, hence it seems safe to conclude that they do not require the vitamin.

With the exception of the clones from one collection (EU 10), all of the European clones belonging to varieties 4 and 6 require more nutrilites than the wild type. Preliminary tests indicate that some (EU 40, EU 149, EN 118, EN 131, EN 132) require all seven additional nutrilites, whereas one (EU 147) needs only glycine, choline, and biotin; and another (EN 120) requires glycine, proline, biotin, and choline. The American 4 and 6 varieties, as well as the one exception noted above, grow on the standard complete medium quite unlike their European counterparts.

DISCUSSION

The results of this study reveal some observations concerning the breeding behavior and nutritional requirements of *T. pyriformis* which may have evolutionary implications.

Regarding the breeding behavior, there seem to be close affinities between those European clones which mate well with the American variety 6 testers even though certain irregularities appear. When the European 6/I (EU 10, EU 40, EU 147, EU 149) and European 6/II (EN 120, EU 143) are crossed no viable offspring result; but when European 6/II is crossed with American 6/I offspring are recovered, although when these are inbred lethality is 100%. No other cross between the American 6 and the European 6 is possible—this is the sole link between the 2 variety 6's. The fact that this cross was possible seems to relegate this group to variety 6, rather than a separate variety. Certainly the vigorous mating with first generation viability dictates a close relationship. This could be interpreted to mean that the American and European clones originally possessed the same gene pool but with time have accumulated a sufficient number of lethal genes to permit partially successful con-

jugation. In other words, the aging European variety 6 (composed of two mating types) is beyond the point of being capable of producing viable offspring, yet the gene flow between the European and American variety (composed of three mating types) is common enough to allow for mating and viable progeny to be produced between the aging European 6/II and the still younger American 6/I. One is inclined to believe that variety 6 was distributed throughout Europe and America in past geologic time and is now showing genetic separation as a result of geographical isolation.

The fact that the European variety 6 clones fail to produce viable offspring is not surprising because many clones within American varieties mate vigorously for years after they are incapable of producing offspring. Aging has been observed in many of our clones in the past few years. In the present study two strains (EU 64-1—3/V and EU 56-14—6/II) mated satisfactorily in October of 1959, but failed to conjugate when tested a year later. Both clones still possess their micronuclei. No effort was made to obtain viable offspring from these strains when they first mated. It is likely that they will ultimately lose the capacity to divide asexually and will be terminated.

Aging is also common in other species of *Tetrahymena*; for example, in lines of *T. rostrata* where autogamy has been prevented, aging is observed(5). Since autogamy does not occur in *T. pyriformis*, aging without conjugation is a normal process which usually results in the death of the line. The exception is among certain amicronucleate strains which have been in culture for over 30 years with no apparent signs of aging. These clones are sexually inactive and may be considered genetically dead(22).

In spite of low progeny viability it seems likely that the vigorous mating observed in these crosses indicates close affinities. It is always possible that this is a case of intervarietal mating, in which the European clones are actually a new variety. Intervarietal mating has

never been observed in our laboratories, although Orias(20) reports mating between varieties 6 and 8 with good progeny viability (75%) when the cells are grown in bacterized water. However, when the clones were grown axenically in proteose-peptone medium no mating occurred. Since all of our crosses are made with washed cells grown in such media, mating would probably not occur. During the past several years we have, on occasion, mated clones directly from bacterized water and have never observed intervarietal mating. It is possible that the mating reported between varieties 6 and 8 is an isolated case. A nutritional requirement for mating may be more general than is presently known in *T. pyriformis* and a detailed study of this phenomenon is certainly indicated.

The outcome of the nutritional studies of the European clones revealed some unexpected results. Our earlier studies, in which several thousand clones were tested for their nutritional needs, indicated that *T. pyriformis* was remarkably consistent in its requirements. The only variations uncovered were the ability of a small number of clones to survive without serine or pyridoxine(8,9). It came, therefore, as a surprise to learn that many of the European clones required nutrilites in addition to those required by the wild type and strain E. With the exception of one group (EU 10) all of the clones studied possess nutritional requirements that vary from those of the wild type. All clones in variety 10 from England fall into a uniform nutritional pattern, none requiring thiamine, but identical otherwise to the wild type. Clones in both variety 4 and 6 require additional nutrilites as compared to their American counterparts. Those belonging to variety 4 grow on nothing less than 5 additional amino acids (alanine, glycine, proline, aspartic acid, glutamic acid) and 2 vitamins (biotin and choline). Those in European variety 6 are variable, some (EU 40, EU 149) requiring all seven additional nutrilites; whereas others are less demanding, EU 147 needing only glycine and choline, and EN 120 requiring alanine, proline, biotin, and choline.

It will be observed that all clones belonging to variety 4 are from England, are of the same mating type (III), and have the same nutritional requirements. Even though collected from different habitats, they could well be from the same population owing to the fact that they came from the same drainage system. Clones belonging to European variety 6, on the other hand, are widely distributed coming from both England and the continent of Europe, where there are no natural communicating waterways. However, in such studies one must consider the possibility of transporting ciliates during fish breeding operations. Since their nutritional requirements are highly variable one is in-

clined to believe that each has been isolated for a long time in its present environment.

The data seem to support the thesis that there is little or no correlation between nutritional requirements and breeding pattern. For example, the two groups of clones, EU 147 and EU 149, taken from different canals in Amsterdam, Holland, possess the same mating type, yet are nutritionally quite different. It would appear that these two characteristics probably have arisen independently and are unrelated.

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