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Tetrahymena pyriformis from Several Pacific Islands and Australia*

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SYNOPSIS. From a total of 223 diverse habitats located in Australia, New Zealand, Fiji, Hawaii, Japan, Hong Kong, and the Philippines, 144 yielded *Tetrahymena pyriformis*. These collections were taken from a latitude of 35° N. to 35° S., from sea level to 4500 feet, and temperatures ranging from 15° to 30°C. From these habitats 2300 clones were isolated, of which approximately 800 were examined for micronuclei and 450 tested for mating type.

Two distinct interbreeding populations were isolated from the Australian collections. They failed to mate with any of the 10 known varieties and are therefore designated as varieties 11 and 12. No sexually active clones were found in New Zealand, Japan or Hong Kong. However, clones belonging to variety 9, previously found only in Colombia and Panama, were isolated from habitats in Hawaii and Fiji. This evidence suggests that possibly variety 9 is confined primarily to the tropics. There seems to be no other correlation of varieties with land mass.

Representative clones from each habitat were established in axenic culture, all of which grew well in proteose-peptone. When first tested, a number of mutants were found among the 66 clones screened for their nutritional requirements. Some grew without thioctic acid or niacin. Several required aspartic acid and one required biotin and proline in addition to the 18 nutrilites required for strain E. No pattern could be found which related the mutants to a particular region. Examination of the mutants one year later revealed that the synthetic capacity for both niacin and thioctic acid had been lost and that those clones requiring aspartic acid had acquired the capacity to make the amino acid.

A NUMBER of distribution studies of *Tetrahymena pyriformis*(2,7,9) have been conducted in an attempt to detect differences in cross-breeding behavior and nutritional requirements which might point to a pattern of evolutionary changes, perhaps dictated by some types of barriers. Clones have been established from diverse habitats on land masses covering a broad geographical range.

Collections previous to this study made in the United States, Europe, Central and South America demonstrated 10 varieties, each with two or more mating types, revealed by cross-breeding experiments. Serine and pyridoxine mutants were found in the American collections(4,5), whereas mutants requiring nutrilites in addition to the 18 required by strain E were found in the collections from Europe(2).

This study deals with an analysis of collections made in the fall of 1960 from Australia, New Zealand, Fiji, Hawaii, Japan, Hong Kong (Kwangtung Province) and the Philippines. With the completion of this study, *T. pyriformis* have been examined from all the continents except Africa.

MATERIALS AND METHODS

The procedure for collecting and maintaining samples varied

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only slightly from that previously described(2,7). The ciliates were tentatively identified from field collections and mailed to the University of Michigan in sealed plastic capillary tubes. Approximately 20 clones were then isolated from each of these cultures. Each clone was designated by its country, habitat number, and clone number, following the system adopted earlier(7). In this study, as in previous publications, the terms *clone* and *strain* have been used synonymously. Since *T. pyriformis*, unlike *Paramecium aurelia,* does not undergo autogamy, this seems to be justified. Presumably a clone retains its characteristics during vegetative reproduction since there is no opportunity for recombination of genes. It has become routine to use strain designations for those clones which are retained in our permanent collection. These strains possess characteristics, such as mating type or nutritional requirements, which are of interest and may prove of value in future investigations.

In this study approximately 2300 clones from 138 different habitats (6 were lost) were maintained in 16 mm test tubes on *Aerobacter aerogenes* suspended in double glass-distilled water at pH 6.5-6.8(2). These cultures were refed monthly by pouring off approximately one-half of the old bacterial suspension and replenishing with fresh bacterized water. The cultures were initially incubated at 25°C for two days and then stored at 15°C. All clones were finally rendered axenic with antibiotics, employing the same procedure as in earlier studies(2,7). They have been maintained since in our stock medium(2). The presence or absence of a micronucleus was determined by the Feulgen reaction.

Representative clones from each habitat were mated against two mating types from the 10 existing testers as described previously(2). The one exception was variety 5 where only one mating type was used, the other having been lost some months ago. Random cross-breeding tests of nonreactive clones were made with those from different habitats within each country and between countries to identify new varieties and mating types.

Representative micronucleate clones from most habitats for which the variety and mating type could be determined were tested for their nutritional requirements. However, in a few instances nonreactive micronucleate clones were tested in order to discover any unusual mutants that might have been included from certain land masses such as Japan and Hong Kong. Cells in logarithmic growth phase were washed and tested by the omission technique against the 18 nutrilites that support the growth of strain E and/or the wild type(2). A clone was designated as a mutant if it could be subcultured through seven transfers of 0.08 ml of culture medium for each subinoculation. In doubtful cases loop inoculations were made to insure the exact requirements of the mutant in question. It has been our experience that a 0.08 ml subinoculation, at least for the first few subinoculations, permits the likelihood of selecting a mutant more readily than by using only a loop. Apparently the sudden transfer of only a few organisms to deficient medium prevents survival whereas a larger number brings about sufficient adjustment of the medium to permit survival of the ciliates. Later subinoculations can then be successfully made with a loop.

Clones that failed to grow in the complete medium were tested on media containing seven additional nutrilites (alanine, aspartic acid, glutamic acid, glycine, proline, biotin, and choline). All others grew on the complete medium containing the 18 nutrilites that support strain E. These were classified as wild types.

Whenever there was any doubt about the identification of a clone, silverline preparations were made according to the method of Chatton and Lwoff (see Corliss, 1). This was necessary in identifying varieties 11 and 12 and many of the nonreactors. There was always the possibility of including closely related species which could only be ruled out by careful staining procedures.

RESULTS

One hundred forty-four (65%) of the original 223 fresh water samples collected from a variety of habitats in Australia, New Zealand, Fiji, Hawaii, Japan, Hong Kong (Kwangtung Province), and the Philippines contained *T. pyriformis*. The habitats included streams, pools, rivers, ditches, creeks, swamps, springs, and stock ponds. Samples were taken from 35° N. to 35° S. latitude at elevations from sea level to 5400 feet and at temperatures ranging from 15° to 30°C.

Table 1 is a summary of the source, number positive for *T. pyriformis,* micronuclear condition, sexual reactivity, and the classification of the clones as to mating type and variety. The results are described below according to the land masses from which the collections were taken.

Australia

Since the collections were made from bodies of water along highways in the vicinity of and between metropolitan areas, the data have been condensed for convenience into three general regions, corresponding to the three provinces in which the habitats were located. To give the exact location of each collection would be cumbersome and add little, if anything, to the value of this report. Moreover, owing to the importation of fish into Australia from time to time, it seemed prudent to collect samples, in so far as possible, near the source of the stream where it was unlikely that stocking operations had taken place. This reduced the possibility of including *T. pyriformis* that were not indigenous to Australia. The same procedure was followed on other land masses wherever possible.

The total Australian study included water samples taken from 133 habitats along the eastern seaboard and some distance inland. Of these, 94 (70%) contained *T. pyriformis*. Sixty-one percent of the clones isolated from these habitats were amicronucleate. Two hundred three micronucleate clones were isolated from these habitats, none of which conjugated with any of the 10 known varieties from other parts of the world. However, by random combinations among themselves, two new varieties 11 (three mating types) and 12 (four mating types), were found. Occasionally both varieties were present in the same habitat. The distribution of representatives of these varieties according to province is described below.

New South Wales: Collections were made in the vicinity of Sydney, particularly from small streams which originate in the mountains. In general, the

TABLE 1

Sample numbers	Location	No. & % positive samples	No. of habitats tested	No. of clones stained	No. & % mic clones	No. & % mixed clones	No. of clones mated	No. & % reactive clones	Variety	Mating type
AU 1-105	Australia (New S. Wales)	78 (74%)	62	393	81 (21%)	77 (20%)	236	83 (30%)	11 12 11 & 12	I, II, III I, II, III I, II, III
AU 106-123	Australia (Queensland)	9 (50%)	7	57	19 (33%)	4 (7%)	42	14 (33%)	12	I-IV
AU 124-133	Australia (N. Terr.)	7 (70%)	6	67	7 (10%)	15 (22%)	18	0	—	—
NZ 1-19	New Zealand (N. Island)	9 (45%)	8	73	2 (3%)	6 (8%)	10	0	—	—
NZ 20-44	New Zealand (S. Island)	12 (48%)	7	36	3 (8%)	13 (36%)	15	0	—	—
FI 1-4	Fiji (Viti Levu)	2 (50%)	2	2	—	—	77	39 (50%)	9	III
HA 1-3	Hawaii (Oahu)	3 (100%)	2	24	5 (21%)	0	26	5 (19%)	9	III
JA 1-12	Japan (Honshu)	7 (58%)	5	40	3 (7%)	5 (12%)	4	0	—	—
HK 1-14	Hong Kong (Kwangtung)	6 (43%)	6	45	7 (15%)	7 (15%)	11	0	—	—
PI 1-13	Philippines (Luzon)	11 (85%)	11	82	17 (21%)	9 (11%)	15	3	12	(see text)

water samples were taken as near the source of the stream as feasible. The habitats examined were small tributaries of the Parramatta River west and north of Sydney and of the Woronora River south of the city. Collections were also made in the vicinity of Sutherland and Cronulla; both areas are located south of Sydney. In all, twenty habitats were examined in the Sydney area.

Nine samples were collected along the Hume and Federal highways between Sydney and Canberra. Several trips were made from the latter city extending as far as 80 miles south into the Snowy Mountain region, west into the mountainous (4500 feet) area near Brindabella, and northwest on the Barton Highway to Harden. Forty-seven collections were made on these trips in the vicinity of Canberra.

Samples were then taken along the Pacific and New England highways north from Sydney to Newcastle and Armidale. A side trip of about 100 miles east of Armidale to Grafton included mountainous country, consequently, most of the collections were taken from small tributaries of the Wollomombi and Bellingen river systems. Twenty habitats were examined from the Armidale region. The remaining collections in New South Wales were taken along the New England Highway from Armidale to the border of Queensland.

Seventy-four percent of the collections in New South Wales, representing 105 different habitats, contained *T. pyriformis*. Owing to death in transit or after their arrival in Ann Arbor, representative ciliates from 62 habitats were actually examined. Of these, only 20 (30%) yielded sexually active clones, 79 percent of which turned out to be variety 11 and 21 percent variety 12. One of the habitats, 14, contained both varieties. All three mating types were found in variety 11 whereas only three of the four mating types of variety 12 were present. The varieties were randomly distributed over the areas studied, demonstrating no pattern that was discernible.

The nutritional studies revealed, for the first time, strains of *T. pyriformis* that grew without niacin (Au 13-4 and 14-11) when first tubed. Growth was poor but continuous through 7 subinoculations; hence it was likely that these ciliates could synthesize this vitamin at minimal levels. Moreover, other strains such as Au 3-4, 19-6, and 1-2x required aspartic acid (in addition to the 18 nutrilites required by strain E). The latter strain also required biotin and proline, while Au 3-4 required biotin in addition to aspartic acid. As with the varietal distribution, there seems to be no correlation of nutritional requirements with the location of the habitats. As will be pointed out below, these presumably well defined mutants underwent biochemical changes during a year of test tube culture, which may point up environmental influences on nutritional requirements.

Queensland: Collections in this province include those taken from streams along the New England Highway from the border between New South Wales and Queensland to Brisbane, and then in the vicinity of the latter city. Several short trips were taken to the

north, west, and south of the city during which collections were made from small tributaries, most of which drained into the Brisbane River. In all, 18 habitats were sampled, of which 9 (50%) contained *T. pyriformis*.

Four habitats produced reactive clones, all of which belonged to variety 12. One of the habitats, a reservoir near Point Talburpin, was the only sample to yield clones belonging to mating type IV.

One nutritional mutant, Au 114-8, was found in these collections. It required aspartic acid in addition to the 18 basic nutrilites required by strain E. This character was lost in subsequent culture.

Northern Territory: All collections in this province were taken south of Darwin for about 50 miles on the Stuart Highway to Barry Springs. Collections were made from 10 different habitats, all of which were tributaries of the Adelaide river system. Seven (70%) of these habitats contained *T. pyriformis*. All of the samples were nonreactive for mating type and therefore not screened for nutritional requirements.

New Varieties

The two new varieties discovered in Australian waters were assigned the numbers 11 and 12. Variety 11 was found to contain mating types I, II, and III; variety 12 includes an additional mating type, IV. Of 97 Australian clones which were determined for variety and mating type, 62 percent were variety 11 and 38 percent were variety 12. None of the micronucleate nonreactive clones conjugated with any of the ten known varieties, consequently it seems unlikely that any of these varieties occur in Eastern Australia and perhaps on the entire continent. Moreover, cross-breeding tests with the micronucleate nonreactive clones from Europe, the American continents, and other Pacific islands were negative. Hence, insofar as this study is concerned, varieties 11 and 12 seem to be confined to Australia with the possible exception of those few poorly reactive clones from the Philippines (see below).

The morphology of both varieties is similar to that of other varieties. The ciliary rows are somewhat fewer (16-18) than in most varieties (17-23), which is also true of all of the ciliates collected on the South Pacific land masses. Since the rows of cilia vary considerably within a clone, this low number cannot be taken as an identifying morphological character. Several minor physiological characters have been noted which may turn out to be valuable in diagnosis. The refractory period (after 24 hours' starvation) is longer in variety 11 (4-6 hours) than in variety 12 (2-4 hours). Conjugation is out of synchrony in variety 11 and the conjugants remain attached for 24-48 hours whereas in variety 12 mating is well synchronized and the conjugants separate in less than 24 hours. Viability in both varieties is low, being 3-4 percent in 11 and less than 2 percent in 12. No growth pattern differences have been observed between the varieties. There may well be slight physiological differences which have been overlooked in routine culturing and testing procedures.

New Zealand

Water samples were taken from 44 habitats in both the North and South Islands of New Zealand. Of these, 21 contained *T. pyriformis*, most of which were amicronucleate and therefore nonreactive.

North Island: Collections from this island were first made along the highway extending south from Auckland to Hamilton and Rotorura, the thermal area. This is gentle rolling country with numerous streams and small ponds. The thermal pools, even those that were below 30°C, were devoid of *T. pyriformis* as well as most other protozoa. The second area of study was in the mountainous region north and east of Wellington where most of the samples were taken from the small tributaries of the Hutt River. Some samples were taken from habitats over 2000 feet in elevation.

Of the 19 habitats studied from the North Island, 9 (45%) contained *T. pyriformis*, 8 of which were cloned. Three percent of the 73 clones were micronucleate. Ten representative clones were cross-bred with all 12 varieties; none were reactive.

South Island: All of the collections on the South Island were taken west and north of Christchurch from tributaries of the Waimakariri and Ashley rivers. These streams flow gently over this relatively flat land which is a part of the Canterbury Plain. Samples were taken from 25 habitats, 12 (48%) of which contained *T. pyriformis*.

Of the 36 clones stained for nuclear condition, 8% were micronucleate. None of these clones were reactive with the 12 tester mating types. Nutritional studies revealed a mutant, NZ 40-6, which did not require thioctic acid when tested in 1962. A year later it had lost its capacity to synthesize this vitamin. The mating type of this clone is unknown as it is nonreactive.

Fiji

Collections, on the island of Viti Levu, were taken from small mountainous streams that intersected the road from Suva to Nadi via Koro Levu. *T. pyriformis* appeared in two of the four habitats examined. The clones isolated from one habitat were amicronucleate whereas those from the other proved to belong to variety 9. During a single test, the clones reacted sporadically, mating with only some of the variety 9 testers. It was only by using a series of tests that

all mating types but 9/III were finally eliminated. Reactions with the testers in each case were definitely positive. No new variety 9 mating types were discovered.

Of the reactives, two (FI 1-1, 1-2) were nutritional mutants, growing satisfactorily without thioctic acid when tested in 1962. However, tests a year later revealed that these clones required the vitamin.

Hawaii

Collections were made from three mountain streams on the island of Oahu, near Honolulu. These were the only habitats examined. They were from the Nuuanu, Kalihi Valley, and Manoa rivers. The samples were taken approximately halfway up the mountainside near the source of the streams. *T. pyriformis* was found in all samples, but those from the Manoa River were lost in transit. Samples from the remaining two habitats were cloned and stained to determine their nuclear condition. The clones from the Nuuana River were both micronucleate and amicronucleate, while the clones from Kalihi River were all amicronucleate.

Mating type tests with 26 clones from the Nuuana River showed that 5 clones were variety 9, mating type III. Reactions of the Hawaiian clones were similar to those of the Fiji clones. Three of the reactive clones were tested for their nutritional requirements and two proved to be mutants. One, HA 1-6, did not require thiamine and another, HA 1-3, grew without thioctic acid in 1962. Here again, in 1963 it required the vitamin.

Japan

Collections in Japan were made during January, 1961, from roadside ditches, rice paddies, and small streams along the main highway between Tokyo and Nikko. This is relatively flat terrain with much standing water and many small streams. Seven of the 12 habitats (58%) examined contained *T. pyriformis*. Ninety-four clones were isolated from five of the samples (two of the samples died before they could be cloned). Clones from three of the habitats proved to be amicronucleate and the remaining two habitats contained both amicronucleate and micronucleate clones.

The micronucleate clones were cross-bred with all twelve known varieties. None reacted, hence these probably are either nonreactors or belong to a new variety. Nutritional studies in 1962 revealed one mutant, JA 4-5, which did not require thioctic acid, but in 1963 it would not survive without this nutrilite.

Hong Kong

For convenience, these clones are designated as HK, implying that they came from the island of Hong Kong. Actually, they were taken from small streams and rice paddies in the mountainous terrain between the city of Kowloon and the border of China in the province of Kwangtung. Therefore, they are actually from the continent of Asia. Most of the streams have their sources in the mountains of China and flow toward the sea.

Six of the 14 habitats (43%) contained *T. pyriformis*. Ninety-five clones were isolated, some of which were amicronucleate, some micronucleate, and some mixed. None of the micronucleate or mixed clones conjugated with the 12 known varieties, nor among themselves. One clone, HK 3-17, did not require thioctic acid in 1962. It was not tested again in 1963 as it was lost in culturing.

Philippine Islands

Water samples were taken from 13 habitats along unidentified country roads approximately 50 miles south of Manila where the terrain is mostly mountainous. The many small streams, originating in the surrounding mountains, are used to irrigate rice paddies. Since the crops are harvested at this time of year (December), the water is confined to ditches, ponds and streams. Eleven (85%) of the habitats contained *T. pyriformis*. Both amicronucleate and micronucleate ciliates appeared in the 184 clones isolated. All but three clones (PI 1-4, 5, 6) of those tested failed to react with any of the 12 known varieties. The response of these three clones (which were all from the same habitat) was limited and sporadic. Only a few pairs reacted during each test. Some individuals mated with mating type II of variety 12, perhaps indicating a distant kinship. Representative clones tested for nutritional requirements indicated that all resembled the wild type.

DISCUSSION

Distribution of varieties. *T. pyriformis* was found in some of the water samples taken from all land masses included in this survey of the South Pacific islands and the continent of Australia. This, together with earlier studies, indicates the ubiquitous distribution of this ciliate. In this respect it resembles other ciliates on which studies have been made(13). Several significant observations have resulted from this study.

The occurrence of variety 9 on Oahu (Hawaiian Islands) and Viti Levu (Fiji Islands) came as a surprise as it had originally been found only in South and Central America(7). These observations extend its range many thousands of miles across the Pacific Ocean. It may occur on other Pacific islands as well.

Since both the Hawaiian and Fiji clones conjugated readily with our testers, which are several generations removed from the originals isolated from the Americas

(8), it would seem that they became isolated in their present sites only recently. Certainly any great time period would have resulted in sufficient gene change to prevent cross-breeding. Unfortunately, no progeny survival studies have been made so it is impossible to say how compatible their genomes are. However, the clones from Fiji and Hawaii conjugate more rapidly among themselves than either does with the testers, suggesting that the island clones have closer affinities with one another than with clones from the mainland. If this is true, it would mean that the ciliates may have moved westward from South America, island hopping perhaps, reaching the Hawaiian Islands first and Fiji at some later time. Such migrations would imply that they were carried by some animal that transported a water supply and that could only be man. However, it also is possible that *T. pyriformis* could be transported during violent air disturbances such as hurricanes.

It has been assumed, based on laboratory evidence, that *T. pyriformis* cannot tolerate sea water, hence must be transported in the free-living state. This may not be entirely true. There is the possibility that resistant cysts form at some stage in the life cycle which could remain desiccated for long periods of time and thus be transported from island to island as has been true of other animals and plants. Efforts to produce cysts under laboratory conditions have failed. However, Hurst(10) identified cysts in variety 2 which he claimed were induced to excyst. These appeared in bacterized cultures. They have never been seen in our axenic cultures of variety 2 or any other variety, even after being subjected to adverse environmental conditions which, it would be assumed, would induce cyst formation. If cysts do form in nature, the matter of transportation across great distances would be immeasurably simplified.

As one might expect, the continent of Australia seems to harbor *T. pyriformis* which are genetically isolated from other varieties that have been studied so far. They all fall into two varieties, designated 11 and 12, which show no reproductive affinities with any of the other 10 varieties. With the exception of a few weak reactors from the Philippines, they seem to be confined to the continent of Australia. As *T. pyriformis* occasionally shows cross-reactions between varieties(11), it is possible that the weak mating of the few Philippine clones with variety 12, mating type III, may be such a rare instance. If, on the other hand, these clones did actually belong to variety 12, it is possible that they were imported with fish transplanted from Australia. The ciliates were collected from habitats at low elevations near the city of Manila which would be the ones most likely to be used for fish planting operations.

Concerning the distribution of the two varieties, 11 occurs almost twice as frequently as variety 12. They are distributed randomly over the area studied, showing no correlation with latitude. The varieties were found together in only one water sample and no more than two mating types of either variety occurred in one sample. Moreover, many habitats yielded only nonreactive or amicronucleate clones. These appeared in nearly all collections along with clones belonging to one variety or the other.

It is to be expected that *T. pyriformis* from Australia are confined to this continent, inasmuch as Australia has been isolated from other continental masses throughout most of its geological history. Continental organisms were able to reach Australia only intermittently from Asia, then were separated from the mainland by an ocean barrier. These conditions have produced many species of organisms which, while still resembling known present or past species, are found today only in Australia. This is probably the situation with varieties 11 and 12. After being cut off from the continental gene pool, Australian *T. pyriformis* evolved into the two genetically isolated "species" we now call varieties.

Micronuclear condition. The collections from the Pacific Islands and Australia, like earlier collections, contained ciliates with considerable variation in the condition of the micronucleus, ranging from many without one at all to those with a small one, presumably haploid, to those with a well-formed, normal micronucleus. The instability of the micronucleus was observed where all three types appeared in a culture derived from a single ciliate (clone). The range of completely amicronucleate clones was from 59% in Australia to 89% in New Zealand's North Island. The average number of amicronucleate clones for the entire Pacific collections was 65%. The remaining 35% of the clones were either entirely micronucleate (18%) or contained a mixture of micronucleates, amicronucleates, and haploids.

The number of amicronucleate clones in this study is about the same as was found in the Central and South American collections (60%) but approximately twice that found in the collections taken from the United States (33%) and Europe (39%). Owing to the limited number of collections, these percentages are not significant in themselves; however, the relatively high percentages of amicronucleate ciliates that appear in all populations examined suggest that they represent a natural component of the life cycle of this ciliate. Moreover, it would seem that there must be some minimal number of sexually active organisms in a population to maintain the species, since amicronucleate ciliates do not conjugate and are, therefore, genetically dead. This number must represent from one-third to two-thirds of the population. It is possible that the minimal number of sexually active members

in a population does not drop below approximately one-third for maintainance of the species.

Aging is a characteristic of *T. pyriformis* populations as it is in other ciliates(12). This type of population aging must be distinguished from that which occurs during the growth cycle where physiological and morphological changes occur which completely disappear upon subinoculation to fresh media(3). In the latter type, the genes are not affected and the potency of the individual cells is retained. Population aging, as considered in this study, includes genetic changes that are inherited from generation to generation and may ultimately destroy the ciliate line. This type of aging consists of a period of immaturity following conjugation, then a period of sexual maturity during which successful mating occurs, and finally a period of senility which may terminate the ciliate line. The appearance of amicronucleate ciliates in a culture is one of the first signs of senility. The clones in this study containing both amicronucleate and micronucleate cells may represent a transitional stage between sexually mature and completely amicronucleate ciliates. Apparently the single cells from which these clones were derived had already entered the period of senility when they were first isolated. Continued culturing has accentuated the process, possibly more rapidly than it would normally occur under natural conditions. Undoubtedly the rapid growth in rich culture medium is rarely, if ever, simulated in nature. Hence, it may be that the aging process is greatly accelerated under laboratory conditions.

Rather marked variation in the numbers of amicronucleate clones was found in collections taken from the North and South Islands of New Zealand. The former showed 89% and the latter 56%. The high number of amicronucleate clones isolated from habitats in the North Island represents an extreme condition which has not been encountered in any of the other collections. If aging can be correlated with percentages of amicronucleate ciliates in a land mass, it would follow that the populations of *T. pyriformis* on North Island either have become senile more rapidly than elsewhere or are more ancient. This study provides insufficient evidence to come to any conclusions regarding this point.

Selfing (mating within a clone) is thought to be characteristic of aging in *Paramecium*(12) and seems to be true of *T. pyriformis* as well. The number of selfers found in the Pacific collections was remarkably small. Very few clones mated with themselves, whereas collections from other regions of the world demonstrated approximately 5% selfers. If the Pacific collections are as ancient as indicated by the relatively high percentage of amicronucleate clones, the absence of selfers may mean that selfing is not necessarily a step in the aging process.

The Pacific collections yielded many clones of *T. pyriformis* with normal micronuclei that failed to mate with any of the known tester varieties. This was also true of all previous collections. When these nonreactive clones from the Pacific collections and other regions of the world were mixed among themselves, no mating occurred. This means that they are either (1) immature or (2) all of one mating type belonging to an unidentified variety. As to their immaturity, several unsuccessful attempts have been made to mate them, even following many months of subculture, which seems to rule out this possibility. The second possibility seems most likely. If a sufficient number of collections was made from the various land masses, perhaps their mating type and variety could be determined. There is still the possibility that they have become so genetically diverse that even the mating reaction has been lost.

Distribution of nutritional mutants. Tests for the nutritional requirements were made about a year following isolation of the collections from nature. In the meantime, the ciliates were grown for a time in bacterized water and later on proteose peptone under axenic conditions, as pointed out earlier. This observation is mentioned because it may be important in bringing about nutritional changes that apparently occurred during the period of laboratory culture. When first tested, a number of nutritional mutants were found which required more or less than the 18 nutrilites required by strain E. Since they always appeared in habitats where those possessing the wild type requirements were most abundant, one is inclined to interpret them as sporadic mutants having no obvious correlation with environmental factors.

The most frequently occurring mutants were those that grew without thioctic acid when first tested. They were the first clones that have been encountered which survive without this vitamin. Their distribution is widespread: New Zealand, Fiji, Hawaii, Japan, and Hong Kong. This requirement was found in one of the two clones from New Zealand's South Island, two of the four from Fiji, one of the three from Hawaii, one of the four from Japan, and one of the five from Hong Kong. The mutated clones appeared in both nonreactives and in variety 9. The requirement does not seem to be a function of temperature as it occurs from Hawaii to Hong Kong and from Japan to New Zealand. However, it is apparently restricted to the Pacific region as it has been found nowhere else.

The loss of the capacity to synthesize thioctic acid seems to have occurred during the second and third year of subculture in our stock medium under axenic conditions. The tests for nutritional requirements were run several times in 1962 and in each case the ciliates grew luxuriantly through seven transfers without the vitamin. When rechecked in 1963 under the same

conditions, all but one died out on the fourth transfer. The one clone, NZ 40-6, survived the fifth transfer but died on the sixth. It seems that when cultured for long periods of time on our stock medium, the mutants gradually lose their ability to manufacture the vitamin.

A similar situation exists with those clones, (AU 13-4, AU 14-11), that grew without niacin. Both were found in the vicinity of Canberra, Australia, and when first tested grew without niacin, though poorly. They were growing at a low level after the seventh transfer, which would indicate that they could synthesize the vitamin at low levels. A year later, however, they failed to grow after the first transfer.

The four mutants that require aspartic acid in addition to the 18 nutrilites required by the wild type were all found in Australia, three (AU 1-2x, AU 3-4, AU 19-6) from New South Wales and one (AU 114-8) from Queensland. One of these clones (AU 1-2x) also required biotin and proline. The aspartic acid requirement is not correlated with variety as it was found in both 11 and 12. Incidentally, it was also found in the European collections(2), indicating its widespread distribution. Perhaps this requirement is the first step in a series of mutations necessitating more and more nutrilites in addition to the basic 18 required by the wild type.

The aspartic acid requirers resembled the thioctic acid and niacin mutants in that they changed while under culture in the laboratory. When first tested, the requirement was critical and all the mutants died out without the amino acid following the first transfer. However, when rechecked a year later, all four grew through seven transfers in the complete medium without aspartic acid. Apparently, during the period of culture in the stock medium, these mutants reestablished their aspartic acid synthesizing pathways. It may be that the medium itself induces the organisms to adapt to nutritional requirements that are nearly uniform, reestablishing old pathways that in nature were inactivated for one reason or another. In the case of the aspartic acid requirers, such could be true. The niacin and thioctic acid mutants, when first isolated, apparently retained their ancient synthetic pathways, but lost them in culture. Attempts to reestablish these pathways by gradually removing the vitamins from the diet over a period of time have been unsuccessful. It would appear that selection toward those ciliates that need not make the vitamin has proceeded to a point where those with synthetic pathways have been completely eliminated.

Some mutants, however, seem to be quite stable. The mutant which required both biotin and proline (AU 1-2x) has retained the biotin requirement even after over 2 years in culture. Likewise, when thirteen choline requirers found in the European collections were retested after 5 years of subculture, all but one still required the vitamin. They have obviously permanently lost the capacity to synthesize this vitamin.

Another case representing natural selection under cultural conditions was noted in a nonreactive micronucleate clone (AU 76-1) established in axenic culture from estuarine waters along the coast of Australia near Sydney. The sample was taken near the mouth of a river where the water was approximately 20% sea water. When first isolated, this clone grew well in proteose peptone made up in either fresh or sea water. It was kept on both types of media for several months but later for convenience was maintained on fresh water media alone. After a year of subculturing in this medium, the ciliates could no longer tolerate sea water even in high dilutions. During this period, selection toward the fresh water medium apparently had gone so far that small osmotic changes proved fatal. Preliminary tests to readapt this clone to sea water failed.

This one isolated case, which might not be typical, places the manner of distribution of *T. pyriformis* in a new perspective. If this ciliate can adapt to sea water, the matter of distribution to widely separated habitats would be simple indeed. A great deal more work needs to be done before this possibility can be considered as a means of distribution for *T. pyriformis*.

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Miamiensis avidus n. g., n. sp., a Marine Facultative Parasite in the Ciliate Order Hymenostomatida*

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SYNOPSIS. *Miamiensis avidus* n. g., n. sp., a marine facultative parasite, is described. Morphological studies were made on specimens treated with the Chatton-Lwoff silver impregnation technique and living material was examined with phase microscopy. Particular attention was given the infraciliature of the buccal apparatus and its importance to generic assignment in the order Hymenostomatida.

TWO strains, T5 and T16, of a small marine ciliate were reported by the junior author(3,4) in a collection from the local bay waters of Miami, Florida. These protozoa were associated with the bodies of sea horses and were discovered in the course of a search for viruses in marine animals. Originally these two strains of ciliates were referred to as *Tetrahymena* sp., but subsequent morphological studies by the authors have shown that they are generically different from the genus *Tetrahymena* as well as from other genera in the order Hymenostomatida. A new genus and species, *Miamiensis avidus*,[1] is proposed for these protozoa.

It is the purpose of this paper to deal only with the taxonomic status of these ciliates, but attention should be called to their considerable potential value in other research areas. The junior author(3,4) has been able to establish facultative parasitic infections in sea horses and to maintain these ciliates in several types of culture systems.

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[1] The genus name *Miamiensis* was chosen to honor the University of Miami where the ciliate was first isolated and studied. The species name *M. avidus* was chosen because of the greedy feeding habits.

MATERIALS AND METHODS

Two strains, T5 and T16, of *Miamiensis avidus*, were isolated from the original collection and established in several culture systems. Morphological studies were made primarily from silver impregnated specimens. The Chatton-Lwoff silver impregnation technique was employed. Phase microscopical studies were made of the living animals. Nuclear studies were made after staining with haematoxylin-eosin.

GENERAL MORPHOLOGY

Body form and size. The body is generally ovoid in shape with a rounded posterior and a pointed anterior end. The anterior end in organisms of strain T16 appears to be more pointed than in those of strain T5. The length and width of 50 silver impregnated members of strain T5 averaged 31.9 × 16.1 μ. Strain T16 averaged 39.9 × 20.1 μ.

Ciliary meridians. The ciliary meridians vary in strain T5 from 10-12, with the usual number being 11. Strain T16 varies from 10-13 with 10 or 11 or 12 being the usual number, rarely 13. The meridians in both strains are more or less evenly spaced except for the first and last which are thrown over to the right and left around the large buccal cavity. The