Atti Soc. Tosc. Sci. Nat., Mem., Serie B, **92** (1985)
pagg. 1-13, figg. 2, tab. 1.

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THE TANGLED TEMPOS UNDERLYING *TETRAHYMENA* TAXONOMY

Riassunto — *Il problema del ritmo di speciazione sottostante la tassonomia di Tetrahymena.* Il numero delle specie criptiche appartenenti al complesso *Tetrahymena pyriformis* continua ad aumentare ben al di là di quanto atteso, come numero di specie, per le caratteristiche di «outbreeder» generalizzato del complesso stesso.

La molteplicità delle specie, unita all'ampia diffusione della riproduzione asessuale e al parassitismo, suggerisce che nell'ambito del gruppo v'è stata un'adozione secondaria molto diffusa della strategia ecogenetica tipica dello «inbreeding».

Studi di evoluzione molecolare, usando molecole conservative quali l'RNA ribosomale, indicano che vi sono stati eventi frequenti e recenti di speciazione entro il complesso. Questi rapidi e recenti casi di speciazione rendono incerto, ma non eliminano del tutto, l'ipotesi precedentemente avanzata circa la «antichità» del disegno di base del complesso *Tetrahymena.*

Abstract — The numbers of cryptic species of the *Tetrahymena pyriformis* complex continues to increase, far beyond the species multiplicity expected for a generalized outbreeder. The species multiplicity, combined with widespread asexuality and parasitic associations, suggests an extensive secondary adoption of the ecogenetic strategy of inbreeding specialization. Studies on molecular evolution, using conservative ribosomal RNA molecules, indicate frequent recent speciation events in the complex. The rapid recent speciation within the complex renders uncertain, but does not totally discredit, previously expounded views concerning the antiquity of the basic tetrahymena design.

Key words — Molecular evolution /speciation / *Tetrahyma.*

HOW MANY SPECIES?

In a somewhat unsystematic survey of the systematic literature,

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I recently counted some 33 described species of the genus *Tetrahymena* (Table 1). This is a respectable number of species for a genus that wasn't even christened until 1940 (FURGASON, 1940), and whose original type species, *T. gelei*, was later ruled illegitimate (CORLISS, 1953, 1970, 1973). Even so, one can argue with reasonable plausibility that the naming of *Tetrahymena* species is only well begun.

TABLE 1 - Species of the genus *Tetrahymena*, including some recently named ones.

The *T. rostrata* Complex
- *T. rostrata*
- *T. limacis*
- *T. corlissi*
- *T. stegomyiae*
- *T. rotunda* [a]
- *T. bergeri*

The *T. pyriformis* Complex
- *T. setosa*
- *T. chironomi*
- *T. dimorpha* [b]
- *T. pyriformis* cryptic cluster

Micronucleate species
- *T. thermophila* (Syngen 1)
- *T. americanis* (Syngen 2)
- *T. borealis* (Syngen 3)
- *T. hegewischi* (Syngen 5) [e]
- *T. pigmentosa* (Syngen 6, 8)
- *T. canadensis* (Syngen 7)
- *T. tropicalis* (Syngen 9)
- *T. hyperangularis* (Syngen 10)
- *T. australis* (Syngen 11)
- *T. capricornis* (Syngen 12)
- *T. sonneborni* (Syngen 13) [e]
- *T. nipissingi* (Syngen 14) [e]
- *T. nanneyi* (Syngen 15) [d]
- *T. malaccensis* (Syngen 16) [d]
- *T. asiatica* (Syngen 17) [d]

The *T. patula* Complex
- *T. patula*
- *T. vorax*
  - *T. vorax* (sensu stricto)
  - *T. leucophrys* [c]
  - *T. caudata* [d]
  - *T. silvana* [d]
- *T. paravorax*

Amicronucleate species
- *T. pyriformis* (sensu stricto) (Phenoset A)
- *T. ellioti* (Phenoset B)
- *T. furgasoni* (Phenoset C)
- *T. lwoffi* (Phenoset E)

a LYNN et al. (1981)
b BATSON (1983)
c WILLIAMS et al. (1984)
d SIMON et al. (1985)
e NYBERG (1981)

Particularly noteworthy is the fact that 19 of the species belong to the '*Tetrahymena pyriformis* complex' — the most extensively studied group in the genus. Most of the species of this complex are 'cryptic species', sibling species of such similarity that their distinctions escaped the scrutiny of earlier workers. The demonstration of species-grade differences was based initially on breeding tests, and on the idea that the evolutionary unit is the species defined as a Mendelian population. Although many of these species are morphologically indistinguishable, even after breeding tests have identified them, they are unquestionably fully isolated genetic species with little or no introgression and, at least in some cases, separate evolutionary histories of considerable length (CORLISS and DAGGETT, 1984). The question of how long the species might have had independent existence, and the time of the appearance of the first tetrahymena-like ancestor we will return to later.

One reason for believing that the named species of *Tetrahymena* constitute a small fraction of those in nature is that the job of identifying them is very tiresome, and becomes more so as the number of named species increases. Breeding tests are pretty awful taxonomic tools. To use them one must maintain a collection of living reference strains representing all the previously named species. And one must mix unknown strains with representatives of all those species under circumstances appropriate for mating in the species. New species can be named only after extensive study, when two or more new strains produce normal F1, F2 and backcross progeny when mated among themselves, and when they fail to mate with and/or to produce viable progeny with all other sets of strains that are fully compatible within their sets.

Not only do breeding tests require laborious investigation, but the tests are often applicable to only a fraction of the wild strains collected. Many wild tetrahymenas are persistent selfers that mate massively whenever their food supply runs out, regardless of whether they are mixed with some other strain. Since isolated pairs from such matings almost invariably die, the meaning of the mating is hard to ascertain, and the systematic status of the strain is beyond diagnosis with breeding tests. Many other tetrahymenas, perhaps half of all isolations made, are amicronucleate. Although laboratory derived amicronucleates are very rarely able to divide (KANEY and SPEARE, 1983), and even to mate, wild amicronucleates have not been observ-

ed in conjugation. An amicronucleate strain cannot be classified by breeding analysis.

Breeding tests are burdensome and applicable to only a fraction of the things that need to named. Moreover, though they can sometimes demonstrate 'sameness', they are almost useless in measuring degrees of difference, or in organizing species according to any phylogenetic principle. Only when criteria based on molecular diversification are applied can these difficulties be surmounted.

The most useful molecular tests used thus far for assessing relationships in *Tetrahymena* are those based on the electrophoretic mobilities of enzymes that can be visualized with specific dye coupling. Isozyme variation needs to be calibrated, however, in order to be applied in species discrimination. How much isozyme variation occurs within a species? How different do two populations have to be in order to be called different names? Comparisons of species that have been established by breeding tests permit one to scale isozyme variations. BORDEN et al. (1977) reported that individuals within the same breeding group (= syngen = species) were usually alike in at least 2/3 of their isozyme mobilities. This generalization has held reasonably firm for the enzyme systems commonly used. MEYER (unpublished) has recently made 15 pair-wise comparisons of collected strains of *T. pigmentosa* and found two similarity coefficients below 0.67; one of these was 0.61 and the other 0.63. *T. pigmentosa* is the most heterogeneous species of the complex and encompasses syngens 6 and 8, which can be tricked into mating under special circumstances. The low coefficients were between subspecies.

By knowing how much isozyme variation was commonly found within species, BORDEN et al. (1973, 1977) were able to analyse populations that could not be mated. They demonstrated that amicronucleate strains were different from all the known breeding species, but that they fell into several discrete classes (phenosets), as discrete in their isozymic peculiarities as were the breeding species. The number of 'isozyme species' that might exist in nature is hard to estimate. Most amicronucleates have been discarded by collectors, and only those well established as laboratory personalities (GL, E, W, etc.) have been given names. Enough wild amicronucleates have been studied, however, to show that very similar amicronucleates can be collected from widely scattered locations and that some have zymograms similar or identical to those of micronucleate strains; every population is not unique.

My not-very-well-informed guess is that the number of amicronucleate species is as large as the number of breeding species, and that the total of all kinds of species in the complex could run into the hundreds if anyone cared to invest the effort. One must ask, however, whether the *Tetrahymena* situation is 'normal' for ciliates. Are all 'taxonomic species' of ciliates as speciose as those in the genus *Tetrahymena*?

Why so many?

Thirty years ago *Tetrahymena pyriformis* was believed to be an 'ubiquitous' generalized ciliate broadly adapted to the consumption of bacteria in fresh (and brackish) waters all over the face of the earth (Corliss, 1953; Elliott, 1973). Because its simple modified cylinder is so far removed from the baroque elegance of *Euplotes* or *Entodinium, Tetrahymena* has been considered primitive, even prototypic, a model not far removed from the design of the first ciliate ancestor (Orias, 1976). With Sonneborn's (1957) seminal hypothesis concerning the significance of breeding systems in ciliates, *Tetrahymena* — in the form of *T. thermophila* seemed to have the diagnostic features of an outbreeding generalist (Nyberg, 1974; Dini, 1984): a long life cycle, a long period of sexual immaturity, an absence of autogamy, effective resistance to environmental stresses, multiple mating types, and inbreeding deterioration in the laboratory.

But now I am not so sure. The very number of species is bothersome. Generalized outbreeding organisms are not expected to be highly speciose, but to maintain dispersed panmictic populations capable of dealing with a wide variety of ecological opportunities. As an example of an organism with an outbreeding strategy, Sonneborn used *Paramecium bursaria.* This organism has a long life and multiple genetically determined mating types, it lacks autogamy and resists inbreeding. The *P. bursaria* complex seems to be limited to four sibling species of wide distribution. In contrast he posed the *P. aurelia* complex with its relatively short life cycle, its binary karyonidal mating type system and autogamy. This complex is much more speciose with at least 14 geographically more restricted species.

In *Tetrahymena pyriformis* 15 breeding species have been named and the number possible may be limited only by the patience of the investigator. We show that many other species within the com-

plex have totally abandoned the recombinational lottery that drives the genetic economy of the outbreeder. An amicronucleate species — like the most stereotypic inbreeder, must rely soley on mutational variety to surmount environmental challenges. The permanent selfing strains may have transformed conjugation from a means of enriching the genetic mixture of the species into a device for regulating population density. Thus, though *Tetrahymena* has many superficial marks of an outbreeding economy, serious consideration must be given to the possibility that many members of the genus may have turned an ecogenetic corner, abandoned an exploratory life style, and retreated into some exploitative adaptations in which their outbreeding specializations had become a burden.

The observations that precipitate this heresy come not only from the inconvenient multiplication of *Tetrahymena* species, but also from persistent reports that tetrahymenas are not always the clean-living fresh water denizens that we have admired so much. *T. chironomi* (Corliss, 1960) takes its name from the chironomid larvae that it infests. *T. rotunda* (Lynn et al., 1981) appears as a parasite in the haemolymph of another dipteran of the genus *Simulium*. In what must be one of the more bizarre reports in a somewhat dullish business of species description, we find Batson (1983) discussing the Jekyl-Hyde transformation of the parasitic *T. dimorpha* in another simulid. Not all observers of tetrahymenine parasites of diptera bother to christen the things (Clark and Brandl, 1976), but one can scarcely doubt that tetrahymenas are lodged in lots of places that self-respecting free-living ciliates do not belong. And these places are not all in dipteran larvae. Just this summer Staszek Kazubski forced upon me the unwelcome sight of tetrahymenas jostling each other happily in the renal gland of a snail he picked up under a rock beside a lake in Poland. In this case the *Tetrahymena* was a *rostrata*, but the whole kindred must now be suspect.

The combination of the multiplicity of *Tetrahymena* species, along with the mounting evidence that many are making a living in what is generally judged to be a degenerate and specialized business, strongly suggests that the tetrahymenas are abandoning their traditional outbreeding generality (so much like that of species *Homo*) for the exploitation of specialized and evolutionarily transient niches. If this judgement is correct, the species multiplicity of *Tetrahymena* cannot be projected casually upon other genera of ciliates that have maintained more respectable life styles.

When dit it start?

Isozymes allow us to identify species of the *Tetrahymena pyriformis* complex and to determine which are most closely related (Borden, 1977). Isozyme data provide little indication, however, of the absolute age of the complex. Some species comparisons yield no shared isozymic mobilities in studies of 15-20 different molecules, and similarity coefficients of zero. Although similarity coefficients of zero indicate evolutionary distances of infinity, one recognizes that small sample sizes cannot measure remote evolutionary relationships. Indeed, no *set* of molecules with different evolutionary rates is going to provide any simple relationship between genetic distance and elapsed time. More labile molecules will differentiate populations in a short time, and the more stable molecules will require much longer. A similarity coefficient of 75-80% might develop very quickly, but one might not observe a value of 55-60% until perhaps 10 x as long. The actual relationship between elapsed time and genetic distance depends upon the particular molecules in the set; the more remote events are least capable of interpretation.

Because we have few fossil records for ciliates, and because our molecular chronometers are uncalibrated, we have been free to speculate uninhibitedly about the age of the *Tetrahymena pyriformis* complex. With enthusiasm but insufficient justification, I proposed some time ago that the tetrahymenas were very ancient (Nanney, 1977), and I have elaborated on the theme from time to time (Nanney, 1982), finding considerable circumstantial evidence in scattered data about ribosomal proteins, ribosomal RNAs, mitochondrial and macronuclear DNAs, ciliary proteins, structural proteins, etc. (Nanney, 1984). Unfortunately, none of the kinds of molecular diversity being considered was capable of rigorous calibration, particularly for the remote times under consideration. The tetrahymenas might have had a common ancestor 50 million years ago, or 1000 million. The only real outside limit was the age of the eukaryotes, generally placed at some 1.5-2.0 billion years.

To circumvent this generally unsatisfactory situation, my laboratory has begun to assemble data on molecular variation in the tetrahymenids using more credibly chronometric molecules. Particularly, we have begun collecting comparative data on sequence differences in small ribosomal molecules — the 5S and 5.8S rRNAs. The 5S molecule (Fig. 1) is a linear sequence of some 120

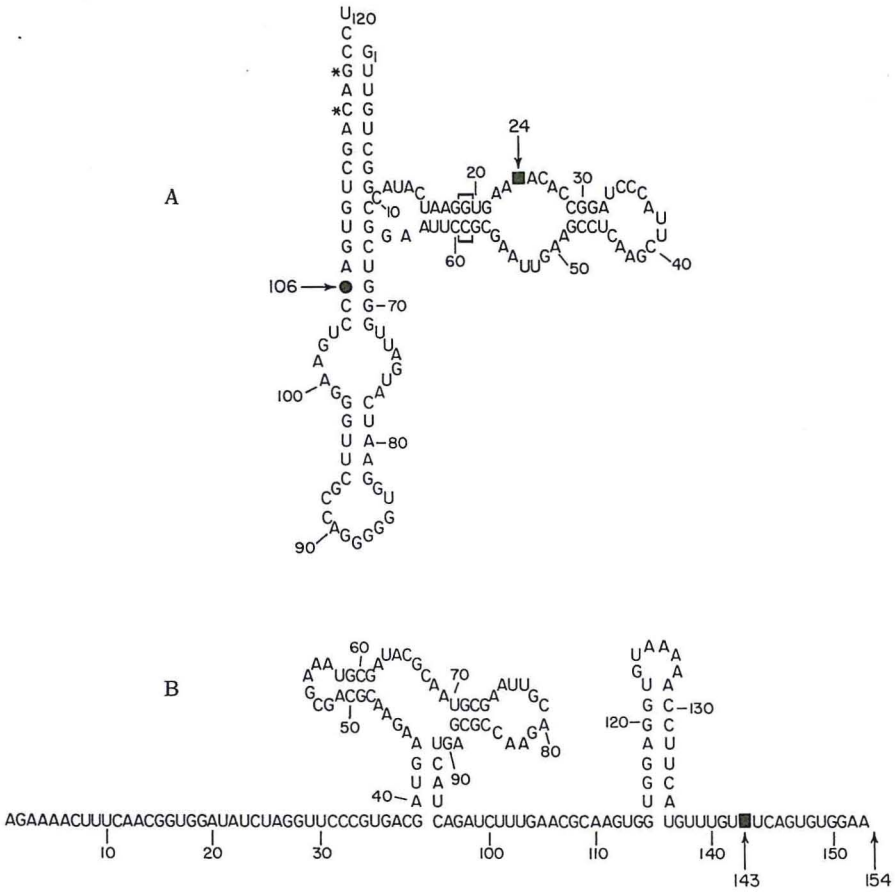

Fig. 1 - A. The 5S rRNA molecule of *Colpidium campylum* illustrating the secondary structure of the molecule with its base paired regions and open loops. The different sequences noted for a strain of *C. colpoda* and one of *Glaucoma* sp. are given. B. The 5.8S rRNA molecule of *Tetrahymena thermophila*, again noting differences observed in the molecules of *Colpidium* and *Glaucoma*. From Van Bell (in press).

ribonucleotides. Because it is one of the components of the 'universal' machinery of molecular genetics, it is present in all life forms. Because it has been very careful with its preserved sequences, the molecule can testify to very remote evolutionary events, including the beginning of the eukaryotes and the origin of the ciliates. The 5.8S rRNA molecule is larger — 154 residues, it is found in all eukaryotes, but is more evolutionarily labile than the 5S molecule.

Both molecules are somewhat constrained by their three-dimensional structures, and by their interactions with other molecules, but nucleotide substitutions can in principle occur at any position in either molecule. Although the two molecules might be mutually constrained in some way, as a first approximation they may be considered as independent evolutionary chronometers.

I will comment first, and only briefly because time and space are finite in ordinary human experience, on the molecular data that are gradually accumulating concerning ciliate origins. 'Universal' molecules, shared by all life forms, and dating back to the 'universal ancestor' (WOESE, 1981) some 4 billion years ago — are not yet very decisive about the structure of the root of the eukaryotes about 2 billion years ago. The uncertainty arises largely from the relative rapidity of the events in the eukaryotic radiation and from the slowness and randomness of the chronometers. The ribosomal RNA molecules (KUMAZAKI et al., 1982; KUNTZEL et al., 1983) and the cytochrome c molecule (TARR and FITCH, 1976; BABA et al., 1981) say that the ciliates appeared early in the radiative explosion, but they provide few details.

A more interesting story may be emerging from the analysis of 'eukaryotic molecules' — those shared by all eukaryotes but missing from archaebacteria and eubacteria. Some of these eukaryotic molecules — histones (GLOVER and GOROVSKY, 1979; HAYASHI et al., 1980; NOMOTO et al., 1982) actins (KAINE and SPEAR, 1982), and calmodulins (YASAWA et al., 1981) representing all the major eukaryotic structures — seem to be unexpectedly deviant in the ciliates. These molecules seem even too distinctive if the ciliates were the first eukaryotic group. Their distinctiveness cannot be explained on the basis of a general evolutionary lability, because it is not apparent in the 'universal molecules', nor do the 'eukaryotic molecules' seem especially variable *within* the ciliates. The simplest interpretation seems to be that these molecules underwent very rapid changes during the origin of the ciliates (NANNEY, 1984). The ciliates — as one of the first successful eukaryotic designs — may have come out of the 'eukaryotic saltation' before their new structures were evolutionarily mature and while they were still malleable, subject to both random and directive evolutionary forces somewhat different from those impinging on the common ancestor of most other eukaryotes.

But back to the ribosomal RNAs and the age of the tetrahymenas. LUEHRSEN et al. (1980) first sequenced *Tetrahymena*

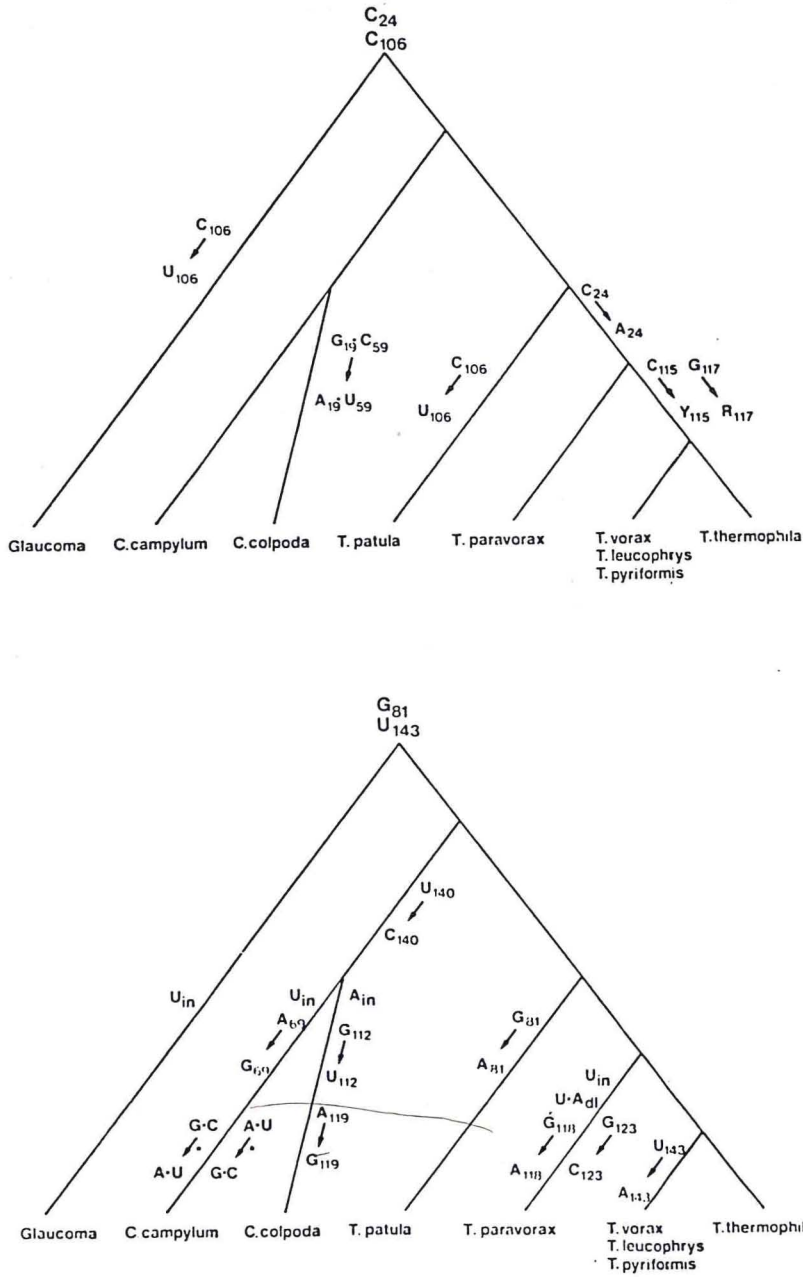

Fig. 2 - Provisional reconstruction of the phylogeny of some strains of *Tetrahymena*, *Glaucoma* and *Colpidium*, based on differences observed in the nucleotide sequences of A. the 5S rRNA molecules, and B. the 5.8S rRNA molecules. From Van Bell (in press).

5S rRNA. KUMAZAKI et al. (1982, 1983) have provided sequences from other ciliates — *Paramecium, Blepharisma, Euplotes* and *Bresslaua.* Van Bell (1985, and in press) working in my laboratory has sequenced several tetrahymenas and close relatives. The data are still being assembled and analysed, and I will not undertake a detailed commentary here. I supply a sample (Fig. 2) of Van Bell's provisional reconstruction of a piece of tetrahymenine phylogeny. It shows that one can make a consistent phylogeny for a sample of species across the genera *Colpidium, Glaucoma* and *Tetrahymena,* using only these small rRNA molecules.

With respect to the age of the tetrahymenine design, however, the most striking fact to emerge from Van Bell's study is the paucity of differences among the *Tetrahymena* species. The 5S RNAs of *T. thermophila* and *T. pyriformis* are alike (and they are identical with those of *T. paravorax, T. vorax* and *T. leucophrys* also); their 5.8S rRNAs differ by a single substitution. Yet one is sexual and the other is amicronucleate; their isozyme patterns are very different. These RNA molecules were chosen because of their conservatism, but they do change, with a rate of something like once in 20 million years. Van Bell's rough estimate of the age of the entire *T. pyriformis* complex is only 30-40 million years, at least an order of magnitude less than my naive early projections. The previously uncalibrated molecular variation among the tetrahymenas becomes much less impressive when confronted with the testimonies of these truly conservative and plausibly chronometric molecules.

The age of the *T. pyriformis* complex, however, is not necessarily the same as the age of the *Tetrahymena* design, which could still be both primitive and ancient. It is still possible that a creature very like modern tetrahymenas was the prototypic ciliate from which all others were derived. The recent proliferation of specialized tetrahymenine species — plausibly tracking different invertebrate hosts and prey — tells us little about the antiquity of the stem line. One can still perhaps entertain the notion of an ancient, structurally conservative, ecogenetically generalized *Tetrahymena* enduring from the distant past into modern times. A rapidly speciating genus, such as *Tetrahymena* now obviously is, must give a dense phylogenetic bush. The more critical questions concern the connections between the tetrahymenas and other ciliates, and between the ciliates and other eukaryotes. For answers we have yet to be patient.

ACKNOWLEDGMENTS

This paper was written while the author was the recipient of an Alexander von Humboldt Senior American Scientist Award, and in residence at the University of Münster. He also acknowledges the support of the United States Public Health Service under Grant GM-07779.

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(ms. pres. il 15 maggio 1985; ult. bozze il 16 dicembre 1985)