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SPECIES CHARACTERIZATION AND SPECIATION IN THE *STYLONYCHIA/OXYTRICHA* GROUP (CILIATA, HYPOTRICHIDA, OXYTRICHIDAE)

Riassunto — *Caratterizzazione delle species e speciazione nel gruppo Stylonychia/Oxytricha (Ciliata, Hypotrichida, Oxytrichidae).* Sono state esaminate alcune caratteristiche morfologiche e in particolare molecolari di species appartenenti al gruppo *Stylonychia/Oxytricha*. Ne viene discusso il loro significato al fine della determinazione a livello di species e per chiarire i rapporti filetici ai vari livelli tra i taxa dei due generi in studio. Viene sottolineato che gli isoenzimi e il modello di bandeggio del DNA macronucleare sono dei buoni marcatori per la determinazione delle specie, mentre gli isoenzimi, combinati alle caratteristiche morfologiche e morfogenetiche, possono dare un ottimo quadro dei rapporti filogenetici che intercorrono fra i membri del gruppo.

Sono riportati poi i meccanismi di isolamento riproduttivo tra le due specie criptiche: *Stylonychia mytilus* e *Stylonychia lemnae*. Sulla base dei dati raccolti viene avanzata l'ipotesi che, nelle aree dove le due specie si sovrappongono, esse mostrano maggior divergenza morfologica e meccanismi di isolamento rinforzato.

Abstract — Several morphological and especially molecular characteristics of the species of the *Stylonychia/Oxytricha* group are described. It is discussed which value they may have for species determination and for the clarification of the phylogenetic relationship of lower and higher taxa. It is concluded that the isoenzyme and the macronuclear DNA banding pattern are good characteristics for species determination. For the investigation of phylogenetic relationship the isoenzymes, combined with morphology and morphogenesis of the cell, are good characteristics.

The isolation mechanisms between the sibling species *Stylonychia mytilus* and *Stylonychia lemnae* are described. It is assumed that in areas where they overlap they show reinforced isolating mechanisms and character divergence.

Key words — Species concept / Biochemical systematics / Ciliates.

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Stylonychia mytilus is well known for over 150 years and was probably already studied 1675 by Leeuwenhoek (see AMMERMANN and SCHLEGEL, 1983). When I started to investigate the mating types and nuclei of this «species», I realized that it consists of two clearly separable species, called «varieties» at that time (AMMERMANN, 1965). They were described recently (AMMERMANN and SCHLEGEL, 1983; STEINBRUECK and SCHLEGEL, 1983). One of them, which shows the typical shape pictured from the first describer, EHRENBERG (1838), kept the old name *Stylonychia mytilus*. The other species received the new name *Stylonychia lemnae*.

In the course of these systematic studies we (my coworkers M. Schlegel, G. Steinbrueck and I) tried to investigate several smaller and not so well known related species. It was, however, not possible to identify most of the collected cells with the aid of the published descriptions and pictures which show only the morphology of the cell. I think the reason for this difficulty is that in earlier descriptions the variation of cell morphology under different environmental influences was never seriously investigated or taken into consideration. Therefore it seems very arbitrary which «variation» was accepted as a variation within a species and which «variation» led to a «new species». The especially unsatisfactory classification of smaller cells as «starvation forms» of larger species is a striking example.

But difficulties do not only exist in species determination. *Oxytricha* and *Stylonychia* were both described by EHRENBERG (1838). The main differences are, according to KAHL (1932) and BORROR (1972): the rows of right and left marginal cirri are confluent (*Oxytricha*) or not confluent (*Stylonychia*) posteriorly. The body is flexible (*Oxytricha*) or firm (*Stylonychia*). However, HEMBERGER (1982) who undertook a more recent revision of the Hypotricha failed to find any ciliates with the main *Oxytricha* characteristics: All «Oxytricha» he saw had no confluent rows. Schlegel and Ammermann (in preparation) also investigated several «Oxytricha» from nature and from different laboratories, and we did not find a cell which showed this characteristics (Fig. 1). The second difference, the flexibility of the cell, is — as I think — not useful, although Hemberger used it as the only mark to distinguish *Oxytricha* from *Stylonychia*.

After these experiences with the morphological characteristics we looked for other features. In the following first part of my article I will discuss the value of some morphological and molecular

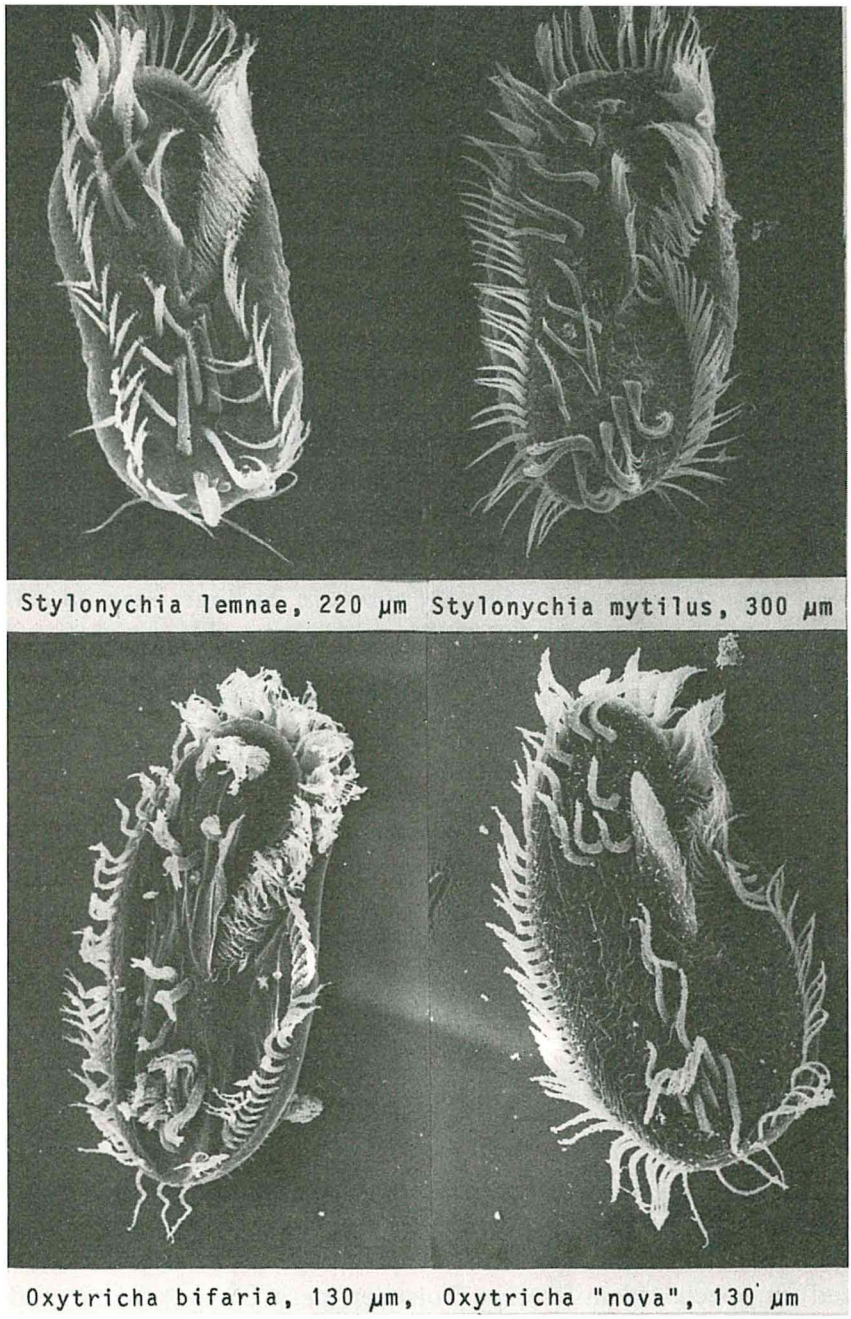

Fig. 1 - Scanning electron microscopic pictures of 2 Stylonychia and 2 «Oxytricha» species. *Oxytricha bifaria* was obtained from Dr. Ricci, Pisa, and *Oxytricha nova* from Prof. Prescott, Boulder, USA. It is apparent that all species have a similar gap between the posterior end of the right and the left marginal cirri. In this gap the three (dorsal) caudal cirri are recognizable, which makes the recognition of this gap in the light microscope difficult (Schlegel and Ammermann, in preparation).

characteristics we found in some species of the *Stylonychia/Oxytricha* group. I will try to answer two questions:

a) Which characteristics are helpful for species determination?

b) Which characteristics are useful for the clarification of «phylogenetic relationship» and for the «construction of phylogenetic trees» of lower and higher taxa?

In a second part of this article I will concentrate on the sibling species *Stylonychia mytilus/Stylonychia lemnae.* I will discuss the question: Which barriers prevent gene flow between both species?

I. *Species characteristics in Stylonychia/Oxytricha*

a) *Morphology*: It was shown that cell size and shape can be used to distinguish the two sibling species *Stylonychia mytilus* and *Stylonychia lemnae* (AMMERMANN and SCHLEGEL, 1983), if the cells are in a comparable stage (I normally compare 24 hours starved cells). With one exception I can determine with reasonable certainty after a look through a stereomicroscope to which species a cultured clone belongs — after 20 years of experience. The exception is the «China subspecies» of *Stylonychia mytilus* (see below). It is, however, impossible to identify single cells in a sample collected from a pond with these criteria alone.

The number of several cirri is constant in all species of this group (e.g. frontoventral cirri, transverse cirri, caudal cirri). Other cirri numbers are correlated with the cell size (see Fig. 2). Therefore I think that in this species group the number of cirri is not a good species character.

It would be worthwhile to make a comparative study of several species with a couple of size parameters as it was done successfully for *Euplotes* by MACHELON and GÉNEREMONT (1984). According to Foissner (pers. comm.) it is possible to distinguish *Stylonychia mytilus* and *Stylonychia lemnae* with this method of biometrical analysis.

b) *Morphogenesis*: HEMBERGER (1982) based his revision mainly on this characteristics. However, the morphogenesis of the *Stylonychia/Oxytricha* species shows no differences which could be used for species identification. Foissner (pers. comm.) found no differences between the species *Stylonychia mytilus* and *Stylonychia lemnae.* Further investigations should clarify how much intraspecific variation occurs and how useful this characteristic is on the genus/family level.

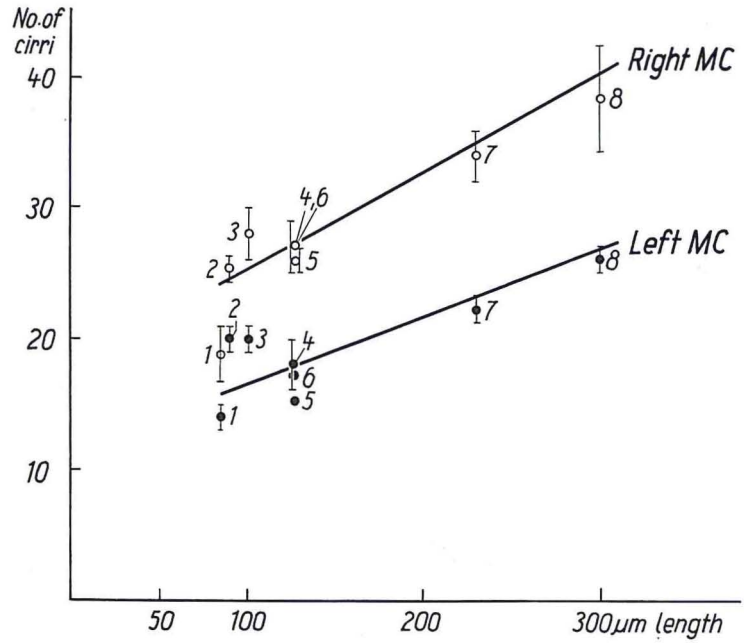

Fig. 2 - Correlation between cell size and number of marginal cirri (MC) of several hypotrich ciliates. The length of the cells was measured after the cells had been starved for 24 hours. The marginal cirri were counted in SEM pictures. 1 *Stylonychia pusilla,* 2 *S.* spec. 1, 3 *S.* spec. 2, 4 *S. pustulata,* 5 *S.* spec. 3, 6 *Oxytricha fallax,* 7 *S. lemnae,* 8 *S. mytilus* (Schlegel and Ammermann in prep.).

c) *Breeding analysis*: To investigate whether two strains belong to one species the best method is to investigate whether both conjugate with each other and whether viable F 1 and F 2 exconjugants occur. Unfortunately this is not easy because of experimental drawbacks. Several species grow excellently in culture, but they do not conjugate, although they conjugate in samples shortly after their collection (from ponds etc.). I have this problem with several *Stylonychia species.* Although I isolated «split-pair» partners I never got more than 1% pairs in crosses of them. Probably some laboratory conditions are good for mass culture, but not good for conjugation. Even if pairs occur in a mixture of 2 strains, it has to be tested whether conjugation between members of both strains or whether selfing happened (Method: Labeling of the cells of one strain). Even if both partners belong to two different strains, there is not necessarily a nuclear exchange. This must also be tested. It is, therefore, obvious that breeding studies can be impossible or rather laborious

or misleading. In the second part of this paper I will give an example. However, for the investigation of species and their delimitation against other species breeding studies are indispensable, but they should always be used together with other characteristics. For the investigation of the phylogenetic relationships of higher taxa breeding studies did not show relevant results until now.

d) *DNA content*: The DNA content of the macronuclei is correlated with the cell size (AMMERMANN and MUENZ, 1982). Therefore the DNA measurements do not reveal more information than the cell size measurements (which are, of course, easier to do). The DNA content of the micronuclei is not correlated with the cell size (1. c.). Its intraspecific variation is, however, rather high, at least in *Stylonychia lemnae* (the only species which has been investigated so far in detail). A study which tries to find the reason for this variation is not yet finished. I think therefore that neither for species determination nor for phylogenetic studies is the DNA content at present a useful characteristic.

c) *Chromosomes*: The micronuclear chromosomes are only visible during meiosis I. During mitosis they are hidden in larger aggregates (DEVIDÉ and GEITLER, 1974). The number of chromosomes is rather high (e.g. n = 150 in *Stylonychia lemnae* (AMMERMANN, 1965)). This high number makes the recognition of «characteristic, species-specific» chromosomes impossible.

During the development of a new macronucleus giant chromosomes develop and are visible for a short time (*S. lemnae*: AMMERMANN (1965), *Oxytricha* sp.: SPEAR and LAUTH, 1976). The rather high number of giant chromosomes is probably the reason for the lack of chromosome maps. But there are, at least in *Stylonychia lemnae,* characteristic regions (with heterochromatin) which can easily be found in all macronuclear anlagen of this species but not in *Stylonychia mytilus.* Therefore I have the impression that the giant chromosome banding pattern could be used to identify species as it is done in Chironomus and Drosophila species. However, to get giant chromosome preparations, it is necessary to have mass cultures which conjugate, and this is sometimes a problem (see previous chapter). Therefore this possibly «good» characteristic was not used until now for systematic and phylogenetic studies.

f) *DNA of micronuclei*: The micronucleus is a typical eucaryotic

nucleus with repetitive DNA, high molecular weigth DNA, etc. If micronuclear DNA is digested with restriction enzymes and then run on an agarose gel, bands are visible. They probably originate from the fragmentation of repetitive DNA sequences (while unique DNA gives only an underlying «background» smear). The banding pattern can be used to distinguish and separate species, although there is some intraspecific variation (STEINBRUECK and SCHLEGEL, 1983). This variation allows investigations and comparisons of populations and subspecies, and it is in agreement with the well-known fact that some repetitive DNA sequences show rapid changes during evolution (JOHN and MIKLOS, 1979). However, in most ciliates which do not have as large micronuclei as *Stylonychia* it is difficult to get enough micronuclear DNA for such studies.

g) *DNA of macronuclei*: The macronuclei of hypotrich ciliates do not contain chromosomes but small DNA molecules which carry, in all cases examined so far, only one gene (review: STEINBRUECK, 1985). If macronuclear DNA is run on an agarose gel, the DNA sequences are separated according to size and form a banding pattern. It is species-specific with only rare intraspecific variation (STEINBRUECK and SCHLEGEL, 1983) and can therefore be used to identify species. For one gel track 5 µg DNA is needed. Due to loss of DNA during preparation we start an experiment with app. 10^6 *Stylonychia lemnae* cells. Separation of macronuclei from the micronuclei is not necessary, because the micronuclear DNA will not run into the gel.

By hybridization of the separated DNA with certain genes or gene products (RNA) it is possible to localize and isolate certain genes. These isolated genes can then be investigated in detail: The length of the genes can be determined, restriction enzymes recognition sites can be localized, etc. Until now only data for few species have been published (e.g. KAINE and SPEAR, 1982; KLOBUTCHER et al., 1984; HELFTENBEIN, 1985). It can be hoped that more data will be published soon, which may allow conclusions about the relationship of some species and their evolutionary history.

All the macronuclear DNA molecules of at least 3 *Stylonychia/Oxytricha* species have the same terminal inverted repeat sequence (review: STEINBRUECK, 1985).

$$\begin{array}{rl} 5'\text{-}C_4A_4C_4A_4C_4 & \text{-------}G_4T_4G_4T_4G_4T_4G_4T_4G_4\text{-}3' \\ 3'\text{-}G_4T_4G_4T_4G_4T_4G_4T_4G_4 & \text{-------}C_4A_4C_4A_4C_4\text{-}5' \end{array}$$

Because the *Euplotes* species have a slightly different telomer sequence (review: STEINBRUECK, 1985), it would be interesting to know this sequence from other genera. Preliminary experiments demonstrated that *Paraurostyla weissei* und *Urostyla grandis* have the same sequence as the *Stylonychia/Oxytricha* group (HELFTENBEIN et al., unpubl.). This shows that the telomeric sequence is too conservative to be a useful characteristic for genus determination.

A method which was used extensively and, as I think, very successfully for phylogenetic studies of Procaryotes and Eucaryotes is DNA-DNA hybridization. One example is a study of difficult bird groups (SIBLEY and AHLQUIST, 1983). This method gives us data about the overall or average similarity (or divergence) of DNA sequences between species. It can be expected that hypotrich ciliates may be especially favourable objects for this method, if mainly active genes are to be compared, because their macronuclear DNA contains no measurable amount of repetitive DNA (which has to be removed in all other eucaryotes before hybridization). Until now, this method was not used to evaluate phylogenetic relationship of hypotrich ciliates. My coworker M. Schlegel has recently started some preliminary experiments.

h) *Proteins*: A number of different proteins were very successfully used to study the phylogenetic relationship between different organisms (e.g. the amino acid sequence of hemoglobin and cytochrome). The necessary methods are, however, quite laborious. The investigation of isoenzymes is much easier and has therefore become quite popular for the identification of «difficult species groups». STEINBRUECK and SCHLEGEL (1983) used this method to characterize the two sibling species *Stylonychia mytilus* and *Stylonychia lemnae*. They showed clearly that the isoenzyme pattern is an excellent character to identify species. A detailed comparison of the isoenzyme pattern of several *Oxytricha/Stylonychia* species was done recently by Schlegel (in prep.). Fig. 3 shows the preliminary dendogram generated by numerical treatment of the enzyme data. Since no clustering of *Oxytricha* or *Stylonychia*-species is recognizable it supports the idea that the *Oxytricha/Stylonychia* species belong to one genus. It demonstrates also that this method is useful for the investigation of phylogenetic relationship of lower taxa (species-genus level). But it is probably not applicable to compare higher taxa (above genus level) because isoenzyme gene changes are so frequent during evolution that homology between them is no longer

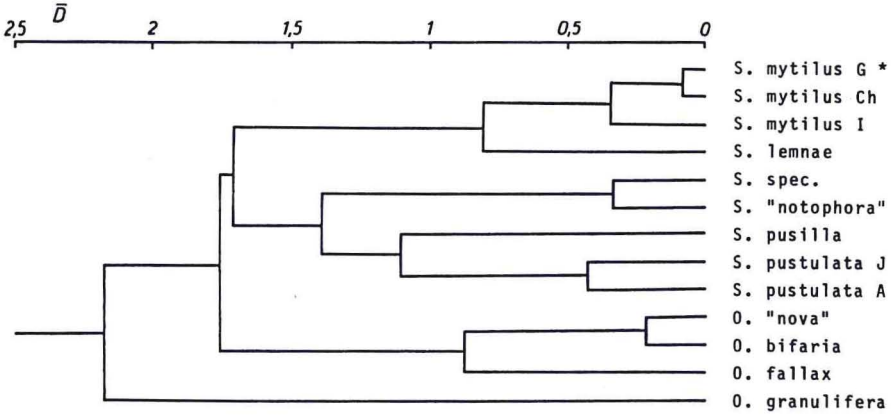

Fig. 3 - A preliminary dendogram showing the relationship between several species of the *Stylonychia/Oxytricha* group. The dendogram demonstrates that the differences between *Oxytricha* species and *Stylonychia* species are not larger than between species belonging to the same genus. All species (except perhaps the rather aberrant *O. granulifera*) should therefore belong to one genus. Capitol letters indicate origin of different strains of one species: G = Germany; Ch = China; I = India; J = Japan; A = Austria. Distance values (D) which are characteristic for different populations and subspecies are in the range between 0.1-0.4 (see *Stylonychia mytilus* «Europe» and «China») (Schlegel, in prep.).

detectable if higher taxa are compared. In these cases it would be better to use and compare more conservative proteins e.g. structure proteins.

Summary of chapter a-h

For species identification in hypotrich ciliates the isoenzyme pattern is the best and easiest characteristic, followed by the more laborious comparison of macronuclear DNA banding pattern.

For the study of the *phylogenetic relationship* of lower taxa (species-genus) the study of the isoenzymes is also the best method. For the reconstruction of the phylogenetic relationship of higher taxa the study of the cell morphology, combined with morphogenesis, is important and indispensable. The publication of 8 different revisions of the Hypotricha during the years 1972-81, all based on morphological and morphogenetic pattern demonstrates, however, that the techniques have to be improved (see Fig. 1) and that there is until now only little agreement about the value of several characteristics (FOISSNER, 1982).

The methods of DNA analysis, especially DNA-DNA-hybridization, are promising for the future.

For nearly all investigations the mass cultivation of the species is necessary. Fortunately many hypotrichs are not difficult to cultivate.

II. *Isolation mechanisms between the sibling species Stylonychia mytilus and Stylonychia lemnae*

The two sibling species *Stylonychia mytilus* and *Stylonychia lemnae* live together in the temperate climate zone of Europe. From North America I have only one clone of *Stylonychia lemnae* (but I have hints that this may be an «illegal immigrant»). From Asia I have only clones of *Stylonychia mytilus* coming from India and from the People's Republic of China. In Europe I found in most ponds I investigated only one species. However, until now I found in 5 ponds both species together. Crossing experiments between all available European clones did not result in any interspecific pair formation. Therefore it can be concluded: The sibling species *Stylonychia mytilus* and *Stylonychia lemnae* are in Europe genetically strictly separated from each other by a mechanism which does not allow pair formation and which is effective *before* conjugation.

The clones I got from Dr. Shi Xinbai (he collected them from two different locations, some 300 km separated from each other, in the Eastern part of the People's Republic of China) are different. These «China» clones are in size and shape between *Stylonychia mytilus* and *Stylonychia lemnae* (AMMERMANN and SCHLEGEL, 1983). Therefore it is impossible to identify them easily with a stereomicroscope. Their isoenzyme pattern shows, however, that they belong clearly to *Stylonychia mytilus,* but they are different from the European clones (see Fig. 3). These results allow me to classify the «China» clones from both localities as a subspecies of *Stylonychia mytilus.* They conjugate successfully (viable F_1 and F_2) with all (European) clones of *Stylonychia mytilus.* To my biggest surprise they conjugate, too, with some (until now 3) populations (all clones of a pond form a population) of *Stylonychia lemnae.* With other populations (5 tested) no interspecific conjugation occurs.

As an example for the relationship of both species I will describe the results of a study of a pond near Tuebingen called Hi. This pond is app. 130 x 70 m large. It has a «reed» zone in which mainly *Stylonychia mytilus* is found. In another area, which contains debris of plants, *Stylonychia lemnae* is found. There is a broad overlapping

zone in which both species occur. In the laboratory all combinations of *Stylonychia mytilus* clones of this pond with clones of *Stylonychia lemnae* of this pond never resulted in interspecific pairing. The «China» clones conjugate successfully (viable F_1 and F_2) with the *Stylonychia mytilus* clones. They also pair with all *Stylonychia lemnae* clones of this pond. It was confirmed that the pairs consist of a partner from each species. However, less than 1% of the progenies of this interspecific crossing survive. It can be concluded that here, too, isolation mechanisms prevent interspecific gene exchange, but the isolation mechanisms work much later, after conjugation started, and the result is in most cases the dead of both conjugation partners.

How can nevertheless some (less than 1%) of the exconjugants survive? Two observations may be relevant for this question:

1) Until now I got three exconjugants of a crossing *Stylonychia mytilus* «China» x *Stylonychia lemnae* «Hi». The isoenzyme pattern, the size and the shape shows that they are pure *Stylonychia lemnae* clones. Probably no nuclear exchange happened. Because they are diploid I suppose that cytogamy in the *Stylonychia lemnae* cells may have happened.

2) I observed the fate of both exconjugants of pairs resulting from a crossing [*Stylonychia lemnae* «Hi»] x [*Stylonychia mytilus* «China», emicronucleated]. From 200 pairs never both partners, but in 20 cases one exconjugant survived. They were clearly *Stylonychia lemnae* haploid clones. I suppose that the nucleus of *Stylonychia lemnae* and the cytoplasm of *Stylonychia mytilus* does not fit together, therefore only one partner could survive.

It appears that the combination of *Stylonychia mytilus/Stylonychia lemnae* genomes and/or the combination of the nucleus of one species with the cytoplasm of the other species fails to give viable hybrid exconjugants. In any case it is apparent that the «China» clones do not have the «usual» isolation mechanisms effective *before* conjugation, but they have a «deadly» isolation mechanism effective *after* conjugation. One can speculate whether the isolation mechanism working before pair formation was not developed from the «China» clones or whether is was reduced. The reason could be the absence of the other species (*Stylonychia lemnae*) in China. This could be another example for a phenomenon which was already discussed by Darwin: The divergence of species characters and isolation mechanisms between species in areas where they overlap (sym-

patric character divergence, Mayr, 1983). To confirm this hypothesis it would be necessary to search in many more ponds in Asia to ascertain whether *Stylonychia lemnae* is really lacking in this continent.

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