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Nuclear Reorganization Variety in *Paramecium* (Ciliophora: Peniculida) and its Possible Evolution

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Summary. The nuclear reorganization pathway of *Paramecium* during conjugation was investigated for 12 morphospecies. For *P. woodruffi* and *P. schewakoffi* the nuclear reorganization was studied for the first time. Several key elements of the process were chosen for the analysis, including „crescent" or „parachute" stage of the micronucleus, number of nuclear products and time of their degradation, and behavior of the old macronucleus. The carried out analysis allowed making dendrogram and topogram of the nuclear reorganization similarity (UPGMA) of the investigated species. The constructed tree did not coincide with the results of our previous molecular-biological analysis in several ways. According to SSrRNA gene sequences, the basal branching pattern of *P. bursaria* and *P. duboscqui* is congruent with some peculiarities of their nuclear reorganization. As an ancient species, they diverged from each other and from *P. putrinum* quite long ago. It seems, the taxonomic species with shorter immaturity period and outbreeding strategy (*P. putrinum*) could show faster evolution rate. For the nuclear reorganization of that species and, probably, for it's mating type system we can expect secondarily modification. General direction of evolution for the old macronucleus changing in *Paramecium* is going, apparently, from the absence of any changing to the macronuclear fragmentation at the earlier nuclear reorganization steps.

Key words: conjugation, evolution, nuclear reorganization, *Paramecium.*

Abbreviations: Con - conjugation, Excon - exconjugants, Ma - macronucleus, Mi - micronucleus, MT - mating type, NR - nuclear reorganization.

INTRODUCTION

Genus *Paramecium* known for 250 years (Woodruff 1945, Wichterman 1986) and as one of the most investigated in ciliates it gave us the model organisms for numerous studies concerning different problems of protozoology, cytology, genetics, general biology and some other. Its wide distribution (for many species cosmopolitan), ease of cultivation, relative large dimensions (80-400 μm), allowed paramecia to become "universal laboratory animals".

Taxonomic species of *Paramecium* preserving similar shape show, however, significant polymorphism of morphology and several biologically important features, e.g. morphology of contractile vacuoles, micronuclei, mating type systems, type of nuclear apparatus, type of reorganization of nuclei during mating processes, and type of endocytobionts. Thus, they are group of organisms unique for comparative biological analysis. Such complex analysis with use of many *Paramecium* species was started not long ago (Fokin 1986, 1997, 2000; Fokin

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and Chivilev 1999, 2000; Fokin *et al.* 1999b; Strüder-Kypke *et al.* 2000a, b). It seems that the obtained data will help to solve some problems important for protozoology as well as for general cytology.

Diversity shown in the course of mating process - Con in different *Paramecium* species, till present, was not the object of comparative analysis, and in some species it was only a little studied or totally unknown. The NR process in the genus is very composed material, useful for studies of NR evolution in unicellular organisms. At present, some data obtained by molecular studies concerning phylogeny of the genus and its position in ciliate system (Strüder-Kypke *et al.* 2000a, b) brings new possibilities for such investigations.

Paramecia disclose amazing for one genus differentiation of several stages of NR in Con, though the general scheme of this process remains almost invariable (Wichterman 1953, 1986; Raikov 1972, 1982; Vivier 1974; Hiwatashi and Mikami 1989). NR contains progamic and metagamic phases. In the first phase, formation of pronuclei (haploid nuclei) takes place during three divisions of Mi (two first of them are meiosis). Exchange of migratory pronuclei and synkarion formation as a result of fusion of migratory pronuclei with the stationary ones end the progamic phase. Metagamic phase is connected with formation of new nuclear apparatus of the cell from products of synkarion divisions. Soon after starting metagamic phase, partners of Con separate, and the further stages of NR process, i.e. formation and development of Ma anlagen, Mi, and degeneration of the old Ma, take place already in Excon.

MATERIALS AND METHODS

The comparative analysis of the course of NR in majority of taxonomical species of *Paramecium* genus was carried out on the basis of the authors own studies (10 species among 12 taken into consideration) and literature data. Analysis was carried out in the following species: *P. bursaria*, *P. putrinum*, *P. duboscqui*, *P. nephridiatum*, *P. woodruffi*, *P. calkinsi*, *P. polycaryum*, *P. caudatum*, *P. multimicronucleatum*, *P. jenningsi*, *P. biaurelia* of the P. aurelia complex, and *P. schewiakoffi*.

Cultures of paramecia were kept in laboratory conditions according to the standard methods of Sonneborn (1970).

Con between individuals from clones representing complementary MT was obtained by mixing about 100 cells in one depression slide (capacity 2.5 ml), 10-12 h after last feeding (medium addition). Besides, self-conjugating pairs (obtained spontaneously or induced) were observed. Selfing was induced by washing paramecia twice in SMB buffer, Dryl's solution or others solutions used in that purpose (Shimomura and Takagi 1984, Yanagi and Haga 1996).

Conjugants and Excon were fixed in different time laps, mainly each 1 or 2 h depending on experiment, by the Bouin's or Carnoy's fixatives, the nuclear apparatus was stained by the Feulgen reaction. The slides were analyzed under magnification x400 to x1000 in microscope Axioscop, Zeiss, Germany, and Amplival, Zeiss, Germany. Alive conjugants and Excon were also observed using device for temporary immobilization (Skovorodkin 1990) under similar magnification and with the application of phase contrast. Data of the studied material (species, clonal, and geographical diversity) are presented in the Table 1.

For comparison of NR course in different species of *Paramecium,* the following characteristic (Jankowski 1972, Ossipov 1981, Raikov 1982) features were chosen: Mi shape in prophase of I-st meiotic division, number of degenerating products of Mi division in progamic phase as well as in metagamic phase, presence of the old Ma fragments, time of beginning of the old Ma fragmentation, time of conjugants separation, number of metagamic cycles, number of Ma anlagen, coordination of beginning of the old Ma fragmentation with time of partners separation (Table 2).

Data concerning NR in the studied *Paramecium* species were noted as presence or absence of the characteristic feature, and then elaborated with the application of cluster and multiple analysis (Fokin and Chivilev 1999, 2000). Classification and ordination of species using the data was made on the basis of Braverman (1965) recalculations of the Euclidean distance without transformation of primary data. The secondary matrix was graphically represented as dendrogram constructed by the using unweighted pair-group method with arithmetic averages (UPGMA) (Sneath and Sokal 1973). Ordination of *Paramecium* species was made by multidimensional scaling (MDS) (Kruskal and Wish 1978) resulting in the arrangement of the species in two-dimensional space (topogram) accounting for dissimilarity of the species' NR traits.

RESULTS AND DISCUSSION

The process of NR in conjugation was observed in the following species: *P. putrinum*, *P. duboscqui*, *P. nephridiatum*, *P. woodruffi*, *P. calkinsi*, *P. caudatum*, *P. multimicronucleatum*, *P. biaurelia* of the P. aurelia complex, *P. jenningsi* and *P. schewiakoffi* (Table 1), while data concerning the NR in Con and after its finishing in *P. bursaria*, *P. putrinum*, *P. polycaryum*, and other than *P. biaurelia* species of the P. aurelia complex - *P. triaurelia* (Przyboś *et al.* 1979) in majority were taken from literature. Literature data concerning NR in *P. caudatum* and *P. multimicronucleatum,* besides author's own observations, were also used for analysis of NR in the species. Rare literature data (usually 1 or 2 papers) concerning NR in *P. duboscqui*, *P. nephridiatum*, *P. calkinsi*, and *P. polycaryum* were also taken into analysis of the process.

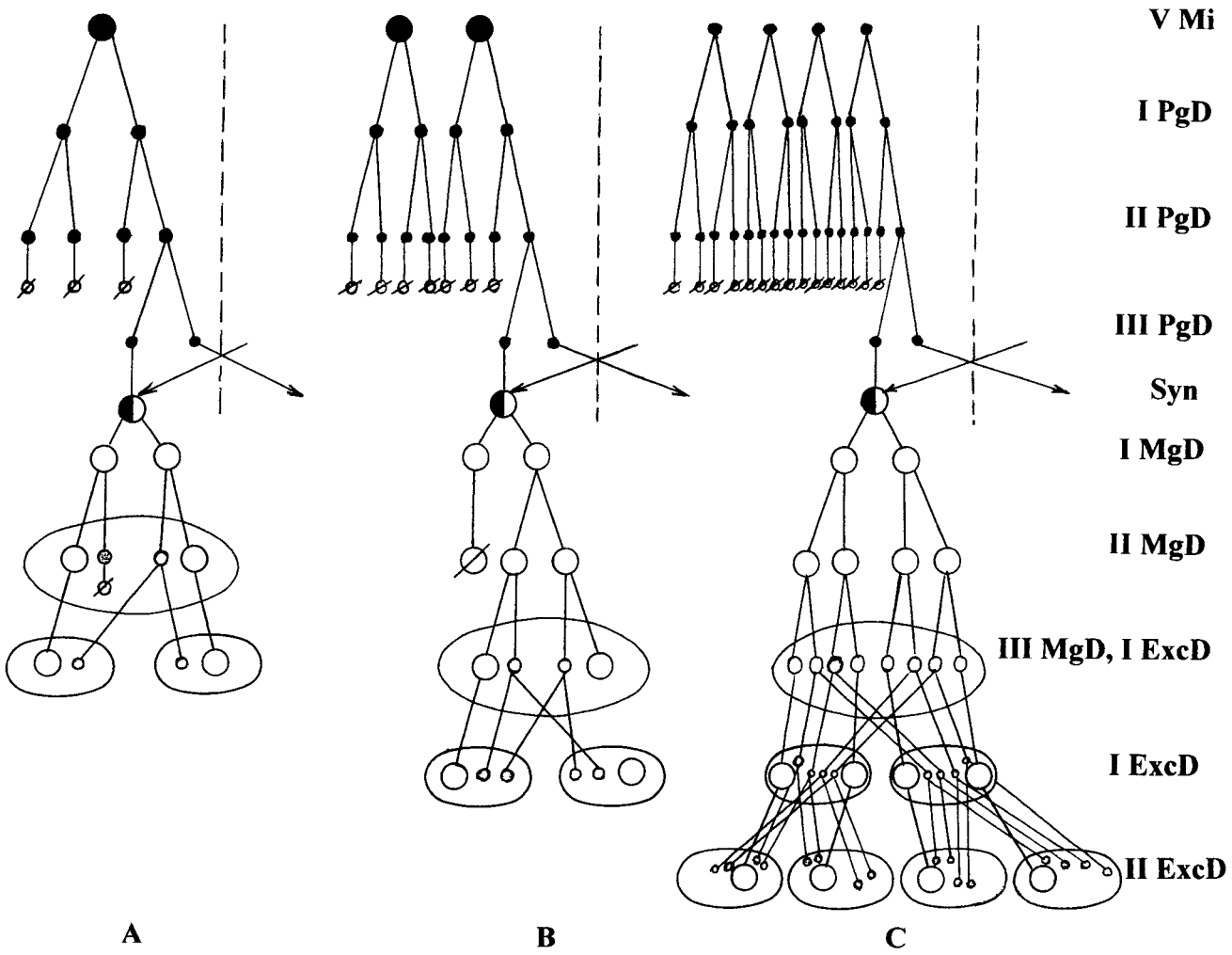
As the main aim of the present paper was comparative analysis of basic stages of NR in *Paramecium* genus, the detailed description of the process in all studied species was neglected. Although, the data on NR in *P. woodruffi* and *P. schewiakoffi* are pioneering, and for *P. jenningsi* is it the first complete description of Con, only the short presentation of processes are given.

Paramecium woodruffi. Process of Con, described by Jankowski (1961), similarly as majority of papers on this species made before 1999, concerned really

Table 1. Cultures of *Paramecium* used for the experimental part of work

Species	Stock	Origin	Type of the nuclear reorganization	Method of *
P. putrinum	JS501-1	Japan, Sendai	conjugation	classical
	JS502-2	Japan, Sendai		
	GK1-1	Germany, Karlsruhe	selfing-conjugation	occasional
	GKz1-2	Germany, Konstanz		
P. woodruffi	BB2-1	Russia, Baltic Sea	conjugation	classical
	BB2-5			
	BB2-10	Russia, Baltic Sea	conjugation	classical
	BB2-12			
	KB3-3	Russia, White Sea	conjugation	classical
	KB3-6			
P. nephridiatum	DZ59-1	Russia, Barents Sea	conjugation	classical
	DZ59-4			
	KB1-5	Russia, White Sea	selfing-conjugation	occasional
P. calkinsi	GN1-4	Germany, North Sea	conjugation	classical
	GN1-9			
	JA1	Japan, Aio	conjugation	occasional
P. polycaryum	UK1-2	Ukraina, Kreimia	autogamy	occasional
	AN1-5	Namibia, Widhook	autogamy	occasional
P. biaurelia	MB3-3	Germany, Münster	selfing-conjugation	induced
	SK2-10	Germany, Stuttgart	selfing-conjugation	induced
P. caudatum	RO1-4	Russia, Lomonosov	conjugation	classical
	RO1-7			
	JY1-8	Japan, Yamaguchi	selfing-conjugation	induced
	BB3	Russia, St. Petersburg	conjugation	occasional
	AP-8	Armenia, Parzlich	conjugation	classical
	AP18			
P. multimicronucleatum	GA1-4	Germany, München	selfing-conjugation	induced
P. jenningsi	JYR-1	Japan, Yamaguchi	conjugation	classical
	JYR-3			
	Sh1-1	China, Shanghai	conjugation	classical
	Sh1-2			
	SA1-7	Saudi Arabia	selfing-conjugation	occasional
P. schewiakoffi	Sh1-37	China, Shanghai	selfing-conjugation	occasional
	Sh1-38			
	Sh1-40			
	Sh1-3		conjugation	classical
	Sh1-38			
	Sh1-40		autogamy	occasional

* - NR were analyzed in conjugants obtained by mixing cells representing complementary mating types (classical method) or in spontaneously received (occasional) or chemically induced self-conjugation (induced).



Figs 1A-C. Schemes of the nuclear reorganization process of *P. shewiakoffi* (**A**), *P. jenningsi* (**B**) and *P. woodruffi* (**C**). V Mi - vegetative micronuclei; I PgD - III PgD progamic divisions; I MgD - III MgD - metagamic divisions; Syn - synkarion; I ExcD, II ExcD - exconjugants divisions

P. nephridiatum (Fokin *et al.* 1999a). In that manner, *P. woodruffi* is one of the less studied species of *Paramecium* (Fokin and Chivilev 1999). Presented course of NR in the species was studied by the authors only in three pairs of clones (Table 1).

Two to five small (3-4 μm) Mi of endosomal type (Fokin 1997) enter I progamic division in synchronous manner. In its prophase „crescent" stage appears. Number of products of the II progamic division differs from 8 to 20 in partners of conjugation. Apparently only one nucleus participates in the III progamic division, and as a result of it two pronuclei are formed. Exchange of migratory pronuclei goes in paroral cone of partners; the stationary pronuclei are also nearby. Beginning of fragmentation of the old Ma starts between the end of II and beginning of III progamic divisions of Mi, and could be seen during exchange of pronuclei as ribbon. All cells observed at the stage of synkarion formation showed disintegrated Ma in ribbon form. Both products of I metagamic division enters II division. At that time, the old Ma appears as round fragments of different size in number 17 to 25 ($\bar{x}$ =20.6 ± 3.7, n=26). Separation of Con partners takes place during or shortly after I synkarion division, further NR goes in Excon. After III metagamic division, the Excon are shorter (about 15% of length), with 8 morphologically similar nuclei, and many fragments of the old Ma. The nuclei, however, are always grouped in 4 on the poles of the cell, in places

Table 2. Main stages of the nuclear reorganization in *Paramecium* species

Species	Nuclear dynamics in conjugants progamic phase, divisions nuclei and its peculiarities			Nuclear dynamics in exconjugants metagamic phase, divisions nuclei and its peculiarities				Ma number anlagen fragments		References
	I	II	III	I	II	III	IV			
P. bursaria	C, 2 (1)	2 (1)	2	2 (1)	2	4^x	-	2	-	Hamburger 1904, Chen 1940a, Wichterman 1948
P. putrinum	P, 2	4 (3)	2	2	4^x	8 (3)	-	4	85*	Diller 1948, Jankowski 1972
P. duboscqui	C, 4	8 (7)	2	2	4^x	8	-	4	2**	Watanabe *et al.* 1996, Fokin 1998
P. woodruffi	C, 4-10	8-20 (7-19)	2	2^x	4	8	-	4	20	Present study
P. nephridiatum	C, 6-8	8-16 (7-15)	2***	2 (1)	2	4	8^x	4	85*	Jankowski 1961
P. calkinsi	C, 4	8 (7)	2	2 (1)	2^x	4	-	2	12	Nakata 1958
P. polycaryum	C, 6-8	12-16 (11-15)	2	2	4^x	8	-	4	19	Diller 1954, 1958
P. caudatum	C, 2	4 (3)	2	2^x	4	8 (3)	-	4	55*	Calkins and Cull 1907
P. multimicronucleatum	C, 6-8	12-16 (11-15)	2	2	4^x	8	-	4	55*	Barnett 1964
P. jenningsi	C, 4	8 (7)	2	2 $(1)^x$	2	4	-	2	34	Mitchell 1962, Przyboś 1975
P. aurelia complex	C, 4	8 (7)	2	2^x	4	-	-	2	33	Jurand and Selman 1969
P. wichtermani	P, 4	8 (7)	2	2	4	8	-	4	?	Wichterman 1986
P. schewiakoffi	P, 2	4 (3)	2	2^x	4 (1)	-	-	2	25	Present study

* - Jankowski's data (1972); ** - products of additional division; *** - according to Jankowski (1961), higher number of pronuclei could appeared; P – the "parachute" stage; C- the „crescent" stage; (1) - number of pyknotic nuclei; 2^x - moment of mates separation; 2 - moment of beginning (skein formation) of the old Ma; ? - absent data

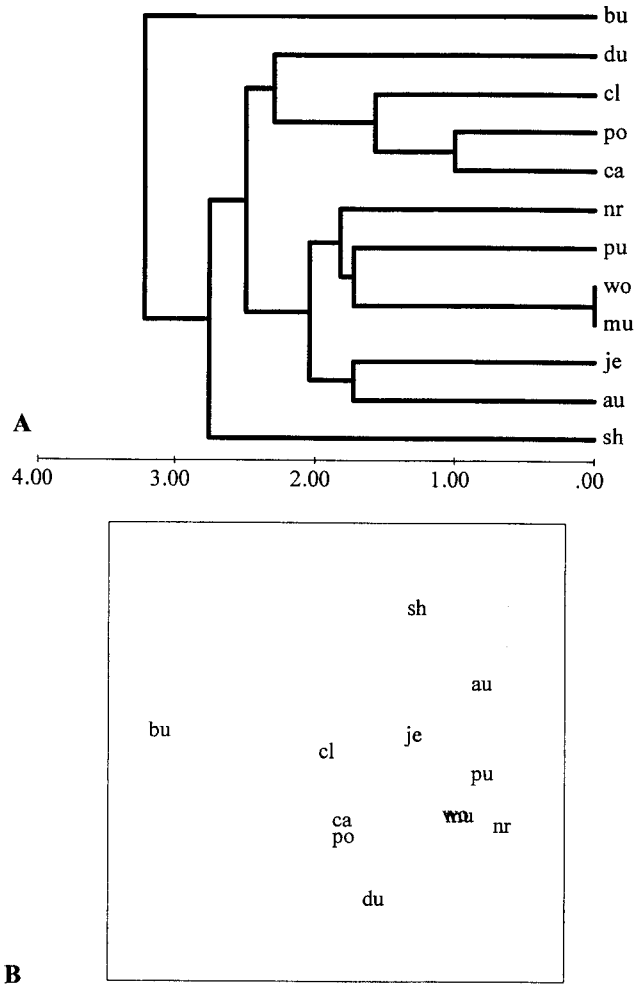


Fig. 2. Dendrogram (**A**) for hierarchical clustering of the nuclear reorganization similarity for 12 *Paramecium* species (UPGMA linking of Euclidean distance similarity) and topogram of non-metric MDS ordination of the features (**B**). au - P. aurelia complex; bu - *P. bursaria*; ca - *P. caudatum*; cl - *P. calkinsi*; du - *P. duboscqui*; je - *P. jenningsi*; mu - *P. multimicronucleatum*; nr - *P. nephridiatum*; po - *P. polycaryum*; pu - *P. putrinum*; sh - *P. schewiakoffi*; wo - *P. woodruffi*

without Ma fragments. Comparison of number of Ma anlagen differentiating from the front group of nuclei and number of Ma anlagen developing from the back group of nuclei (20 cells were investigated) showed slight deviation from the proportion of 4 : 4. Formation of nuclear apparatus, typical for vegetative stage of cell and composed of 1 Ma and 2-5 Mi takes place as a result of two cellular fissions of exconjugants. Ma anlagen are split into daughter cells without their previous division, and Mi anlagen by their mitotic divisions (Fig. 1 C).

Paramecium schewiakoffi. The species revealed in samples collected in November 1999 in China (Fokin *et al.* 2001b). Morphologically it reminds the other species of the "*aurelia*" subgroup, i.e. *P. caudatum*, *P. multimicronucleatum*, *P. jenningsi*, P. aurelia spp. complex. However, the peculiar course of NR of *P. schewiakoffi*, allows easily separating the species from the above-mentioned ones (Fokin *et al.* 2001a). The NR was observed in clones in selfing Con and during autogamy. The conjugating pairs were also regularly obtained by mixing clones representing the two complimentary MT, mature for mating reaction. The course of NR in autogamy occurs similarly like in Con, besides capacity of pronuclei. Autogamy appears in this species regularly in intervals of 8-10 days (16-24 fissions).

Single, relatively large Mi (7-8 μm) of chromosomal type is typical for the species. In prophase of I meiotic division appears „parachute" stage, not „crescent" typical for the majority of *Paramecium* species. Both products of the I-st Mi division enter the II progamic division, and III division enters only one (of 4 Mi) situated near oral zone of the cell. At that time, Ma begins formations of ribbons; the process is finished when exchange of pronuclei takes place. At I metagamic division, the Ma preserves the ribbon form. After that division, the partners of conjugation separate and the old Ma is fragmented. Many round or slightly prolonged fragments ($\overline{x}$ = 25.5 ± 4.7, n=30) of diverse dimensions appear. Both products of I synkarion division start II metagamic division, as a result of it two small morphologically similar nuclei appear on each pole of the cells. The Ma and Mi anlagen were counted in exconjugants (n=40) and some slight deviations from 2 : 2 proportion were found, sometimes Excon showed 3 Ma anlagen and 1 Mi. In Excon (before cell fission) two nuclei develop into Ma anlagen, one nucleus develop into Mi, and one degenerate. Ma anlagen are distributed into daughter cells at the first Excon fission without previous division of nuclei, and single Mi divides mitotically (Fig. 1 A).

Paramecium jenningsi. Con in the species was known since description of the species (Diller and Earl 1958). The whole process of NR in the species was not fully described (Mitchell 1962, 1963), in spite of many investigations carried out on different stages of Con (Przyboś 1975, 1978, 1980, 1986a).

In vegetative stage, two Mi of chromosomal type (Fokin 1997) with diameter of about 5 μm appear. Both Mi enter I progamic division, showing „crescent" stage in prophase. Only one (situated near oral zone of cell) of products of II progamic division do not degenerate. The

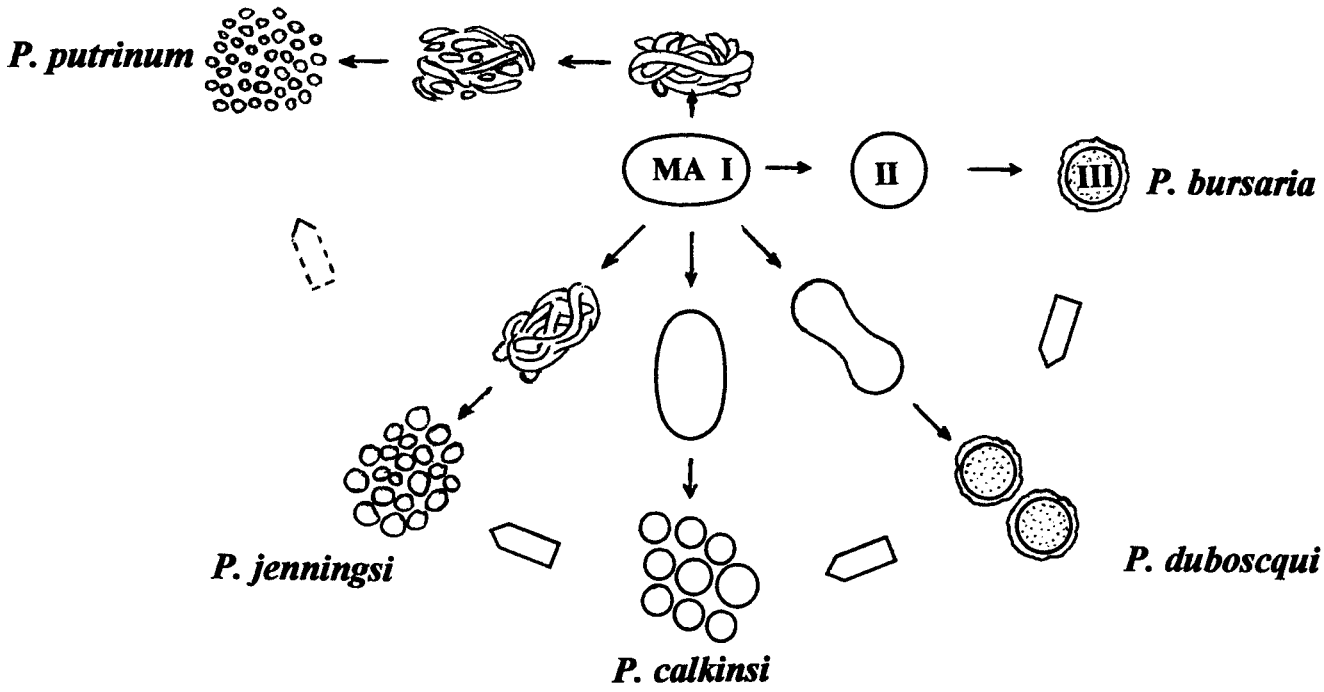


Fig. 3. Scheme of evolutionary changes of the old macronucleus during its nuclear reorganization in the species of *Paramecium* genus. MA - macronucleus; I- progamic phase; II - metagamic phase; III - exconjugant phase

remaining nucleus divides mitotically giving two pronuclei. The process occurs in both partners synchronously. At that time, the Ma loses its round shape and begins formation of ribbons. During synkarion division, the Ma appears as numerous, round or oval fragments, differing in dimensions ($\overline{x}$ = 32.3 ± 4.1, n=16). One of the products of the I synkarion division degenerates, second one enters into II division. The polar pairs of nuclei appear in III division, the Ma and Mi anlagen. Similarly as in the other *Paramecium* species, exconjugants at the last metagamic division are characterized by smaller dimensions and typical form of the cells (it helps to find easily paramecia in that stage of NR between other Excon). At the first fission of Excon, the vegetative form of nuclear apparatus is reconstructed. Both Mi anlagen divide mitotically, Ma anlagen are separated into daughter cells without division (Fig. 1 B).

Similarly, excluding exchange of pronuclei, the process of NR occurs in autogamic individuals. Autogamy is typical for the species, occurs each 7 to 8 days (20-24 fissions).

Other *Paramecium* species. As the peculiarities of NR in the studied *Paramecium* species are presented in Table 2, the basic stages of the process in the remaining species will be analyzed only in comparative aspect. In *P. wichtermani* the process of NR was only shortly described by Nashid (Wichterman 1986), thus there are no information at all concerning some stages of the process. That is the reason for not taking the data concerning the species for the NR comparison.

Comparison of the basic stages of NR in the studied *Paramecium* species allows defining the range of diversity of the process in all morphological species of the genus, existing at present as laboratory cultures (Table 2). This restriction is caused by the fact that some of the undoubtedly valid species, e.g. *P. wichtermani* do not exist as alive cultures in any laboratory in the world, and there is total lack of data concerning Con in *P. africanum*, *P. ugandae*, *P. pseudotrichium*, and *P. jankowski* (Przyboś 1986b, Wichterman 1986). At the same time, the P. aurelia complex composed of 15 biological species, i.e. 14 species (former syngens) named by Sonneborn (1975) and *P. sonneborni* (Aufderheide *et al.* 1983) do not show any differences in the course of NR.

The simplest and sure moment marking the beginning of NR, which we can test, seems characteristic form of prophase of I progamic (meiotic) division of Mi. Among

the investigated species only in three of them, i.e. *P. putrinum*, *P. wichtermani*, and *P. schewiakoffi* the Mi prophase appears as „parachute", in the other 9 species, it appears as „crescent" in different forms (Table 2). Both types of meiotic prophase are widely distributed in ciliates (Raikov 1982, Przyboś 1986c). The „parachute" stage, homologous with zygotene of classical meiosis (Raikov 1982) appears in Karyorelictea, Prostomatea, Nassophorea, Spirotrichea, and Colpodea. In other ciliate groups, mainly in Oligohymenophorea, appears „crescent" instead of „parachute" stage. However, in two species (not free living) of the class, one belonging to Astomatida and second to Apostomatida, the „parachute" was described (Poljansky 1926, Summers and Kidder 1936). Later (Serrano *et al.* 1987) the parachute was found in very common free living peniculine *Urocentrum turbo* as well.

Thus, within *Paramecium* genus stages typical for different subclasses of class Oligohemenophorea can be found what seems very intriguing. On the other hand, relatively few species of Oligohymenophorea were investigated in details karyologically. The situation illustrates state of our present knowledge. In *Frontonia* sp. there are species showing „parachute", and others „crescent" stage (Devi 1961, Perez-Silva 1965, Fokin unpublished), such diversity seems very fundamental.

The other stages of progamic cycle of Mi divisions following prophase of I division, go similarly in majority of *Paramecium* species (Table 2). It is worth to notice, however, some events: in *P. bursaria* one of products of I progamic division always degenerates; in *P. nephridiatum* sometime extra pronuclei appear, later degenerating (Jankowski 1961); in *P. putrinum* great diversity observed in Con is characteristic for the species, there are variants of NR showing behavior of pronuclei and synkarion differing from the most common "amphimixis" type (Jankowski 1972).

According course of the old Ma fragmentation, two groups of *Paramecium* species can be formed (Table 2). One group includes *P. bursaria* and *P. duboscqui,* species without fragmentation, the second group includes species in which fragmentation begins before separation of conjugants (*P. putrinum*, *P. woodruffi*, *P. nephridiatum*, *P. multimicronucleatum*, *P. jenningsi*, the P. aurelia species complex, and *P. schewiakoffi*) or later in Excon (*P. calkinsi*, *P. polycaryum*, *P. caudatum*). Number of the old Ma fragments seems to be specific for the species (Jankowski 1972), e.g. 12 fragments in *P. calkinsi*, in the other species from 19 fragments in *P. polycaryum* to 85 in *P. putrinum* (Table 2). In *P. duboscqui* changes of old Ma in NR have unusual character, Ma do not degenerate like in *P. bursaria* but its extra division takes place during III metagamic cycle (Table 2, Fig. 3). Similar alteration of the old Ma was observed in *Urocentrum turbo* (Serrano *et al.* 1987), which is not a close relative of *Paramecium* (Strüder-Kypke *et al.* 2000b).

It should be stressed that the process of NR was studied, besides *Paramecium*, only in two others Peniculina, i.e. *Frontonia leucas* (Devi 1961) and *F. acuminata* (Perez-Silva 1965). Some details of the cited above descriptions showed that in *Frontonia* spp. appear some variants of the old Ma behavior, similar like in *Paramecium*, e.g. absence of fragmentation in *F. acuminata* (Perez-Silva 1965), extra Ma division (?) before its fragmentation in autogamous *F. leucas* (Devi 1961). Contemporary studies of NR in Con in two *Frontonia* species (Fokin, unpublished) proved existence of variants of old Ma behavior as well.

As a result of fusion of two haploid pronuclei, derivatives of III progamic division, usually in *Paramecium* species the diploid synkarion appears. In some species (*P. bursaria*, *P. putrinum*, *P. caudatum*) several deviations from the statement scheme may be observed, causing polyploidy (heteroploidy) (Chen 1940b, Cheissin *et al.* 1964, Borchsenius and Ossipov 1971, Jankowski 1972). For instance, pronuclei in such cases may be not haploid, so synkarion might be triploid or higher ploidy (Raikov 1972, 1982; Ossipov 1981), more than two pronuclei may be fused or numerous synkarions may be made, as in *P. caudatum* and *P. putrinum* (Jankowski 1972; Raikov 1972, 1982; Ossipov 1981) with further their fusion. Such cases might be fixed in evolution because poliploid Mi could show functional advantage in vegetative stage over diploid one (Fokin and Ossipov 1981). In *Paramecium*, excluding *P. bursaria* (Chen 1940b, Ovchinnikova 1970) such variants are not often cytologically observed.

Differences of NR course during metagamic cycle agree with it's lasting, could be 2, 3, or 4 divisions of synkarion and its derivatives and degeneration of different anlagen in several stages of the process. Partly, such changes appear in exconjugants (Table 2). Majority of *Paramecium* species (11) show 3 synkarion divisions, though as a result of pycnosis (*P. bursaria*, *P. calkinsi*, *P. jenningsi*) of one of two products of first synkarion division, the final number of nuclei is 4. Such number of nuclei also appears in species of the P. aurelia complex and *P. schewiakoffi*, species within two metagamic divisions. In *P. nephridiatum*, according to Jankowski

(1961) (presently, our scanty data do not prove it) pycnosis of one product of I synkarion division takes place causing that the final number of nuclei equal 8 is obtained after 4-th division. That distinguishes *P. nephridiatum* from relative *P. woodruffi* and the other *Paramecium* species (Table 2). Four metagamic divisions of synkarion were described (beside *P. nephridiatum*) only in autogamous NR of *F. leucas* (Devi 1961). In *F. acuminata* (Perez-Silva 1965, Fokin unpublished) only two metagamic divisions appear.

Sometime in a species, the course of Con may be variable depending on individual features of cells (Skoblo and Ossipov 1968, Jankowski 1972). In *P. putrinum* being characterized by composed structure of species (subspecies, syngens) a great variability of NR appears. At present work, the most typical amphimixis type (Jankowski 1972) is discussed only.

In *Paramecium* species, as result of metagamic divisions, 2 or 4 Ma anlagen and similar number of Mi anlagen are formed. Reduction of Ma anlagen number does not appear usually, however sometimes the number may differ. Number of Mi anlagen in some species (*P. putrinum*, *P. caudatum, P. schewiakoffi*) is reduced, by pycnosis of 3 from 4 nuclei (*P. putrinum*, *P. caudatum*) or 1 from 2 nuclei (*P. schewiakoffi*). The reason of degeneration of some morphologically and topologically identical nuclei that looks like apoptosis (Davis *et al.* 1992) is unclear till now (Raikov 1972, 1982; Ossipov 1981; Hiwatashi and Mikami 1989).

Fragmentation of the old Ma has functional character as its fragments do not undergo pycnosis and in some species under suitable conditions, process of Ma regeneration takes place (Jankowski 1961, 1972; Raikov 1982; Ossipov 1981; Fokin 1998). In many of *Paramecium* species (5), the fragmentation starts in progamic part of Con (Table 2), sometime (*P. putrinum*) even during I meiotic division (Jankowski 1972). In *P. calkinsi*, *P. polycaryum*, and *P. caudatum* fragmentation of Ma appears during II or III divisions of metagamic cycle, sometime in exconjugants. Number of Ma fragments is typical for species (Jankowski 1972), though our calculations in *P. nephridiatum*, *P. caudatum*, and *P. multimicronucleatum* showed 35, 46, 48 in sequence, less than cited by Jankowski (Table 2).

Analysis of NR course in *Paramecium* species (Table 2) allows to make dendrogram and MDS topogram of nuclear reorganization similarity for 12 species (Figs 2 A, B). Large number of features taken for analysis makes the obtained figures credible. *Paramecium bursaria* branches basally to all other *Paramecium* species showing definitely a very different features of NR (Fig. 2 A). The other species branching of the tree is *P. schewiakoffi*. It has not so different features of NR in comparison with the other species but its combination looks quite unusual. The position of the two species on the MDS topogram (Fig. 2 B) confirms that the species are located quite far away from the other concerning NR. The next clade includes *P. duboscqui*, *P. calkinsi*, *P. polycaryum*, and *P. caudatum* (Fig. 2 A). Only *P. caudatum* and *P. polycaryum* have quite similar NR since *P. duboscqui* and *P. calkinsi* are located on the border of the group (Fig. 2 B). Two other groups of species apparently have more NR similarity. There are *P. jenningsi* and the P. aurelia complex and, from the other side *P. nephridiatum*, *P. putrinum*, *P. woodruffi*, and *P. multimicronucleatum* (Fig. 2 A). The position of *P. nephridiatum* in the cluster is caused by extra, 4-th metagamic cycle. In other respects it is really similar with the NR of *P. woodruffi*. It seems surprising that NR of *P. woodruffi* and *P. multimicronucleatum* are almost identical (Figs 2 A, B). According to NR, the position of *P. putrinum* which should be considered as an ancient species is astonishing.

As a result of SSrRNA gene sequences comparison in 12 *Paramecium* species the phylogenetic relationships of species was constructed (Strüder-Kypke *et al.* 2000a, b). The genus *Paramecium* seems to be monophyletic cluster of species *P. bursaria* branches basally. The next clade includes *P. putrinum* and *P. duboscqui*, evolutionary very remote from the other species of *"woodruffi"* subgroup, i.e. *P. woodruffi*, *P. nephridiatum*, and *P. calkinsi* which appear as relative species composing a distinct cluster. The position of *P. polycaryum* is not so clear; it may belong to subgroup "*woodruffi"* (more likely) or to "aurelia". The members of "aurelia" subgroup compose monophyletic cluster in which the most related are species of the P. aurelia complex; *P. jenningsi* is very closely related to them; *P. caudatum* and *P. multimicronucleatum* seem more distantly related each other and with the other species of the subgroup.

Using comparative data on diversity of NR course in particular *Paramecium* species correlated with phylogenetic position of the species in the cluster, it seems possible to estimate the evolutionary direction of variation of the NR process in *Paramecium*.

It should be noted that evolutionary specialization proceeds independently in different organelles or processes in the same animal (Jankowski 1972, Corliss

1975, Raikov 1982) and it is called mosaic evolution or evolutionary heterochrony (Jankowski 1972, Raikov 1982). It might be the reason that NR tree did not coincide in several points with the results of analysis on molecular level. However, the position of *P. bursaria* arising from its NR is completely conformable with its phylogenetic one, and this ancient species of *Paramecium* in some traits is similar to *Frontonia*. The other features of *P. bursaria* antiquity (besides the behavior of the old Ma) are prominent symbiosis with *Chlorella* algae and peculiar structure in the Mi mitotic apparatus called by Schwartz (1978) "kinetosome". The typical kinetochores were not found in *P. bursaria* (Lewis 1975, Raikov 1982).

Paramecium putrinum was regarded by Jankowski (1972) as the most primitive *Paramecium* species, very close to *P. bursaria*. However, concerning the NR of *P. putrinum*, the mentioned author proposed, "the nuclear reorganization scheme has been secondary modified in this primitive paramecium ". The morphometric, biological and molecular analyses (Fokin and Chivilev 1999, 2000; Fokin 2000; Fokin *et al.* 1999b; Strüder-Kypke *et al.* 2000b) indicated that this species had separated from the rest of the *Paramecium* species after *P. bursaria*, but not as a sister species. It also shows considerable evolutionary distance from all other species of *Paramecium* except *P. duboscqui* (Strüder-Kypke *et al.* 2000b). The appearance of the „parachute" stage also separates *P. putrinum* from the majority of *Paramecium* species.

Only *P. bursaria* and *P. putrinum* among of the genus have a multiple MT system, the most common in Ciliophora. According to Jankowski (1972) those species inherited the multiple MT system from the ancestral form or forms. Unfortunately, up to present, we have no data on the MT system of the nearest relatives of *Paramecium*, i.e. *Frontonia, Disematostoma, Lembadion*. Miyake (1996) suggested that "the most primitive of the mating type systems is the binary one and ... one of possible general tendencies in evolution of MT is the increase of the number of MT". Following this idea, it should be admitted that in two most primitive species, i.e. *P. bursaria* and *P. putrinum*, the system of MT has been secondary modified.

The idea of secondary modification of NR process in *P. putrinum* (Jankowski 1972) seems true. Only *P. bursaria* and *P. putrinum* are the real outbreeders within the genus *Paramecium* and *P. putrinum* has a very short immaturity period. According to Dini and Nyberg (1993), the species with short immaturity period and outbreeding strategy could show faster evolutionary rate. *Paramecium putrinum* has five different patterns of NR, which mainly correspond, with the different syngens of this species and three morphological subspecies were mentioned for the paramecium (Jankowski 1972). It is likely that *P. putrinum* developed its current NR pattern during evolution of the species.

Morphologically *P. duboscqui* resembles the "*woodruffi*" subgroup of species (Fokin and Chivilev 1999) but its some biological features and results of molecular analysis indicate its position as situated closer to the base of the genus tree (Fokin and Chivilev, 2000; Strüder-Kypke *et al.* 2000a, b). In our both constructions, NR tree and MDS topogram (Figs 2 A, B), the species is located far away from the rest of the "*woodruffi*" subgroup (mainly caused by the behavior of the old Ma, as the other events in NR remind those of the "*woodruffi*" representatives).

Within the "*woodruffi*" subgroup, rather weak similarity can be find, as concerns the NR, between *P. calkinsi* and *P. polycaryum* (Figs 2 A, B). It is supported also by other analyses (Fokin 2000, Fokin and Chivilev 2000, Strüder-Kypke *et al.* 2000b). *Paramecium nephridiatum* and *P. woodruffi* have similar morphology and evolutionary distance between them is very short according to SSrRNA gene sequences (Fokin and Chivilev 1999, Strüder-Kypke *et al.* 2000b). Apparently, the NR process of *P. nephridiatum* (Jankowski 1961) should be reinvestigated, as the data on the fourth metagamic cycle seem not true. The position of *P. polycaryum* within the genus is a bit unstable as concerns morphometric and biological analysis from one side and phylogenetic investigation from another (Fokin and Chivilev 1999, 2000; Fokin 2000; Strüder-Kypke 2000a, b). The species is similar to *P. caudatum* (from the "aurelia" subgroup) according its NR peculiarities but according to SS rRNA gene sequence it cluster consistently with the "*woodruffi*" subgroup.

Paramecium calkinsi has only 2 Ma anlagen, 3 metagamic cycles (caused by degeneration of one of its synkarion products), and a special way of the old Ma fragmentation (as concerns time and number of fragments). The last feature appears also in *P. polycaryum*.

The higher *Paramecium* subgroup, the "aurelia", also does not manifest one type of NR (Figs 2 A, B). The process of NR is similar in *P. jenningsi* and the P. aurelia complex, it agrees with our phylogenetic results (Strüder-Kypke *et al.* 2000b). The different features of NR manifested by *P. caudatum* and *P. multimicronucleatum* correspond to more distant

relationship between the species in comparison with the aurelia and *jenningsi* relationship. Interesting enough that *P. multimicronucleatum* and *P. woodruffi,* which belong to different subgroups but both are rather large in size and have plural Mi, also manifest very similar pathway of the NR.

Paramecium schewiakoffi, apparently, should be placed in the "aurelia" subgroup but its phylogenetic position is not yet settled. The species reminds morphologically *P. jenningsi*, *P. caudatum*, and *P. wichtermani* (at least as the parachute stage is concerned for the last species). Nevertheless, its NR pattern is completely different from all other *Paramecium* species (Fig. 2 A). The key points for its discrimination are: the "parachute" stage in prophase of I-st progamic division of Mi and existence of 2 Ma anlagen, which develop in two metagamic cycles. Its life strategy seems to lead to the high rate of evolution as in the case of P. aurelia complex and *P. jenningsi. Paramecium schewiakoffi* might appeared not long ago. The species was found only in one locality in China but still the huge area of subtropical Eurasia is almost not investigated as ciliate fauna is concerned (Przyboś and Fokin 2000a, b).

As was shown previously, the *Paramecium* species maintain morphological stability being molecularly differentiated, at least as measured by the SS rRNA sequence divergence (Strüder-Kypke *et al.* 2000b). The genus is showing also a variety of karyological characteristics but it is possible to find out in their NR pattern some evolutionary tendency. Behavior of the old Ma could be the example. There are two main patterns of macronuclear degeneration in ciliates, with its fragmentation, which is more frequent (Raikov 1969), and without it. The ability of Ma fragments to regenerate means that they do not undergo the irreversible degenerative changes for a long time (Raikov 1972, 1982). Their functional interactions with the Ma anlagen, i.e. inhibition of the old Ma fragments or suppression of development of the Ma anlagen, indicate that the process is dramatically important for ciliate's cell (Sonneborn 1947, Jankowski 1961, Ossipov and Skoblo 1968, Berger 1973, Raikov 1982, Fokin 1998).

The absence of the old Ma fragmentation is, apparently the primitive feature in the evolution of nuclear dualism (Raikov 1972, 1982; Orias 1986). It seems that the species manifesting the old Ma fragmentation in different stages of NR, developed it in different time during evolution. Summing up our data and literature ones, it is possible to prepare the scheme of evolution of the old Ma behavior in ciliates, using as a model *Paramecium* genus (Fig. 3). Ancient pattern of the old Ma resorption without nuclear fragmentation is demonstrated by *P. bursaria*, that type can be often found in Ciliophora (Jankowski 1972; Raikov 1969, 1972, 1982). In *P. duboscqui* the process is going on with one additional macronuclear division, which could be homologous with the first step of fragmentation as in *F. leucas*. In *P. caudatum, P. polycaryum*, and *P. calkinsi* the fragmentation takes place in exconjugants only, and the number of fragments in two last species is rather small. In the next group of *Paramecium* species, i.e. *P. schewiakoffi, P. jenningsi*, the P. aurelia complex, *P. multimicronucleatum*, and probably *P. wichtermani*, the fragmentation occurs a little before or during separation of the conjugation partners, in the middle of the NR. Evolutionary these species belong to the "aurelia" subgroup. Only three species *P. nephridiatum, P. woodruffi* and *P. putrinum* manifest the old Ma fragmentation at the beginning of NR or in the first part of it. According our analysis, *P. nephridiatum* and *P. woodruffi* are very close related species, which diverged quite recently. A number of syngens of *P. putrinum* with different pattern of the NR and even morphological divergence within this taxonomic species (Jankowski 1972) indicates that we are dealing with a series of quite recent secondary modifications. Thus, general evolutionary direction of the old Ma changes during the NR is from the absence of fragmentation to the appearance of it at the early NR stages (Fig. 3).

Type of Mi I-st progamic prophase, the „crescent" or the „parachute" stage, appear in different ciliates. Further investigations of Oligohymenophorea may bring more examples of species showing parachute stage.

It is likely, that the ciliate divergence occurred after evolution of the functional features of meiosis and fertilization of the nuclear cycle, which enabled a life cycle with haploid and diploid phases (Orias 1986). Short haploid phase and very important beginning of diploid one occur during the NR process in ciliates. As a basic, progamic phase of the sexual process is more stable. Part of the NR stages (mainly during metagamic phase of Con) has a wide diversity in *Paramecium* species. Several of the variations probably reflect some evolutionary stages of the NR development. May be the NR process in *Paramecium* generally has a tendency for shortening (see Fig. 1).

However, taking into consideration the existence of mosaic evolution or evolutionary heterochrony of some features in *Paramecium*, it seems better to use for the

phylogenetic schemes a combination of data obtained from several different traits and by different approaches.

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