

Epigenetic factors in vital functions of the ciliate *Dileptus anser*

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Summary

The article reviews data on serotypes of various clones of the ciliate *Dileptus anser* and results of hybridologic analysis of the mating types features obtained during the long-term studies. The current knowledge on *Dileptus* in terms of epigenetic variation and inheritance is summarized and discussed.

Key words: ciliates, *Dileptus anser*, serotypes, mating types, genetics, non-Mendelian inheritance, epigenetics

Introduction

The genetic features of ciliates define their role as useful model organisms for discovering and understanding the mechanisms of heredity. Early genetic analysis carried out by Sonneborn (1937) showed that the transmission of many heritable sings cannot be fully explained by the Mendel laws. Today nobody doubts that a substantial role in the processes of vital functions of a cell belongs to the epigenetic factors. Epigenetics develops rapidly, and the data on epigenetic variability are continuously supplemented with new important scientific information. Modern views on epigenetic variability are largely influenced by research carried out on ciliates. The study of ciliates allowed, thanks to their unique biology, to discover some new examples of epigenetic heredity. Epigenetics included all inherited changes not related to differences in DNA base sequences (Preer, 1993; Russo et al., 1996). For ciliates, epigenesis can be determined in the observed cases of non-Mendelian heredity.

The ciliate *Dileptus anser* (Rhynchostomata, Litostomatea) (see: Adl et al., 2012) is a common

object not only for protistological but also for the basic biological research. Genetic studies of this ciliate have never been performed until mid-XXth century, which undoubtedly hinders its further use as a laboratory model. In the 1970s, such studies began in the Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS, St. Petersburg, Russia. The clones of dilepti were obtained from the individuals isolated at different times from natural reservoirs in the Leningrad District. Dilepti were cultivated at 25° C in Prescott's inorganic medium and fed with the ciliate *Tetrahymena pyriformis* (Nikolaeva, 1968). This species was a new one for research on genetics and epigenetics of ciliates and highly promising in terms of comparative genetics of these protozoa. For those studies, the characters were selected which were typical for classical genetics of ciliates, namely, serotypes and mating types.

Serotypes in *Dileptus anser*

Serotype of ciliates is determined by immunological methods and results from the presence of a

certain class of proteins distributed on the surface of the cilia and the outer membrane of the cell (Beale and Mott, 1962; Doerder, 1981). These proteins are called immobilization antigens (i-antigens) because when ciliates are treated with homologous immune serum (IS) in a certain concentration, the cells become immobilized. The function of these surface proteins is still unknown. The essential features of the antigenic structure of ciliates are its diversity and variability detected as early as in the 40s of the last century (see one of the first reviews: Beale, 1954). Under different conditions, ciliates of the same clone may express different i-antigens. This genic system operates on the principle of mutual exclusion of the genes, i.e. only one i-antigen can be revealed on the surface of cells at any given moment (Nanney, 1980). In some cases, this rule concerns not only different genes but also different alleles of the gene. Exclusions are extremely rare. When changing the cultivation conditions or under some treatment, the replacement of one surface protein with another one and the cell serotype transformation occurs. Of greatest interest in the serotype system in ciliates are mechanisms of regulation of activity of the surface protein genes that ensure their expression according to the principle of mutual exclusion, as well as a tendency of some established serotype to be inherited in a series of cell generations. These issues have been the subjects of numerous studies (see Bleyman, 1996).

A set of *D. anser* clones was used in our experiments. Immune sera (ISs) were obtained by immunizing rabbits with cell homogenate prepared from mass culture of a corresponding clone. Each of such ISs was considered to be homologous to the cells used for immunization and heterologous to the cells of all other clones. At first, ISs were obtained against two different clones of dilepti. Like other well-studied representatives of ciliates (*Paramecium* and *Tetrahymena*), dilepti demonstrated good ability to induce the formation of specific antibodies after immunization of rabbits with cells of a certain clone of ciliates. Both ISs were characterized by high titers and caused characteristic immobilization reaction when applied to the homologous cells, thus showing the association of the IS specificity with a given i-antigen. Such reactions have been described in *Paramecia* and *Tetrahymena* many times (reviews: Beale, 1954, 1957). Using two antisera, the serotypes of 20 *Dileptus* clones of different origin were identified. Totally, 38 heterologous “cell-to-serum” combinations were tested

(Uspenskaya, 1988). In 33 combinations, there was no reaction with heterologous ISs, and it was clear evidence that they had some other serotypes determined by other surface antigens. However, in 4 heterologous combinations with one of the ISs and in 1 combination with the other one, weak immobilization reaction was registered, indicating some similarity of the serotype in reacting to heterologous and homologous clones. Subsequently, some other clones were investigated, and cross-reactions between the ISs and ciliates cultured at different temperatures were also observed. These observations were made for clones #28 and #29, subclones of each of them being cultured at 25 and 17° C. Two ISs were prepared against two cultures of #5D clone which were cultured at the same temperatures (IS-5D-25 and IS-5D-17). Each IS immobilized only homologous (“warm” or “cold”) #5D cells. Nevertheless, at both temperatures #29 subclones reacted with both of these heterologous ISs obtained against 5D cells. Similarly, both IS-5D-25 and IS-5D-17 immobilized cells of the #28 clone cultured at 17 or 25° C. Consequently, the clones #28 and #29 had the same serotype at both temperatures. Such type of cross-reactions has been described in *Paramecium*.

When discussing the nature of cross-reactions of antisera with heterologous *D. anser* subclones that were cultured at different temperatures and comparing our results with the available literature data, we suggest that the incidence of cross-reactions can be explained in two ways. First, serotype of #28 and #29 clones, which is detected at both 17 and 25° C, may be determined by a single i-antigen related to “warm” and “cold” serotypes of #5D, but manifests itself in a wider range of temperatures. Another possibility is that, contrary to the principle of mutual exclusion, cells of #28 and #29 clones synthesize two different antigens, which are simultaneously expressed at both temperatures (Uspenskaya and Yudin, 1992). Under constant conditions, serotype of dilepti remained unchanged in a great number of agamic cell generations. When testing the above clones a year and a few years later, there was almost 100% immobilization of cells with homologous sera and the lack of cell response to heterologous sera. Testing other clones that had further enriched the laboratory collection revealed only 2–3 additional cases of cross-reactions.

So, the use of even a small number of ISs revealed wide polymorphism of serotypes in natural populations of *D. anser*, and this polymorphism

was maintained during long-term cultivation under constant conditions. To investigate the inheritance of serotypes in *D. anser* during sexual processes, we used two clones, #B and #D, grown from individuals independently isolated from nature. These clones belonged to a complementary mating types I and III, actively conjugated and showed no cross reaction when tested by immobilizing cells with antisera, i.e. showed two different, clearly distinct serotypes that were stably maintained under the same culture conditions. ISs were obtained against these clones. Thereafter, their crossing was made. Cells of one of the two clones were labeled with Indian ink for precise identification and selection of heterotypic pairs. In a paired state the cells were for 20–22 h, and exconjugants were isolated and grown. Unfortunately, it was impossible to see the label in the isolated exconjugants in order to identify their “cytoplasmic” origin.

Exconjugant F1 clones were tested for cell immobilization reaction using ISs raised against B and D cells. The tests were performed after 30 days following conjugation and then after 4 months — when the exconjugant clones reached sexual maturity (Uspenskaya and Yudin, 2000; Yudin and Uspenskaya, 2000). It was found that 1 month old F1 clones reacted with both antisera, i.e. they had a “hybrid” phenotype. Judging by the reaction of immobilization, i-antigens of both “parent” types were often present on the surface of these cells not in equal amounts. At the same time, no regular dominance of one or the other i-antigen was observed: 14 F1 clones reacted more strongly with anti-B IS, and 19 clones — with anti-D. The “hybrid” phenotype persisted during further culturing of the clones up to their maturation. Several clones were tested using the indirect immunofluorescence method and were homogeneous with respect to their serotype.

Thus, we have not seen in F1 neither inheritance of the trait under study according to “maternal” (“cytoplasmic”) type (such as inheritance of serotypes in *Paramecium primaurelia*), nor a situation that could be described as allelic exclusion, nor a situation that would be like vegetative sorting of serotypes in heterozygous clones of *Tetrahymena thermophila*. In contrast, the pattern was the same as the inheritance of allelic serotypes in heterozygotes of *P. tetraurelia*, when there is co-expression of alleles (see Beale, 1954, 1957; Preer, 1968; Sommerville, 1970; Finger, 1974; Nanney, 1958, 1980; Bleyman, 1996). Initially, the genetic nature of

the differences between B and D serotypes was unknown. In the simplest case, this difference could be for just one locus (allelic differences) or for two different loci. The absence of any serological cross-reactions between B and D clones indicates significant immunological differences between the corresponding i-antigens. This might be an evidence of nonallelic nature of the respective serotypes. Phenotype in F1 clones was certainly “hybrid”. If B and D serotypes are allelic, then we are dealing with co-expression of the corresponding alleles; if they are non-allelic, then in *D. anser* there is a violation of the principle of mutual exclusion of different loci controlling surface i-antigens. This challenge could be resolved by receiving F2 progeny and analyzing the segregation of studied traits. When testing exconjugant F1 clones for their mating types, it was found that most of the survivors were fully mature and belonged to mating type II. For this reason, to obtain further F2 progeny, the F1 clones were mated with each of the “parent” clones (backcrossing). It was found that all F2 clones had the same “hybrid” phenotype as F1 clones — there was no segregation for the character analyzed. Some F2 clones from different crosses were tested much later (after 6 and 9 months) and it was found that they stably maintained their “hybrid” serotype (Uspenskaya and Yudin, 2000). Thus, there was a lack of segregation in the second hybrid generation, i. e. the non-Mendelian inheritance of the character. This result can be suggested as a consequence of violations of the rule of gamete purity in the first hybrid generation. Such a deviation from the normal Mendelian behavior of hereditary traits correlates with the hypothesis of the epigenes (Thuraev, 1975, 2010), which is one of the numerous variants of the concept of dynamic hereditary memory (Riggs and Porter, 1996). One of the first hypothetical schemes of epigenetic control in *Paramecium* was proposed just to explain the peculiar features of the genetic control and inheritance of serotypes (Delbrück, 1949). It was this scheme that served as the starting point for all subsequent concepts of this type (see reviews: Olenov, 1965; Golubovsky, 1996; Riggs and Porter, 1996; Russo et al., 1996; Golubovsky and Tchuraev, 1997; and some others). In ciliates that are characterized by nuclear heteromorphism, this scheme apparently becomes more complicated. Changes in gene activities described by the hypothesis of epigenes, should probably occur in the phenogenetically active ampliploid (Raikov, 1996) vegetative nucleus — macronucleus (Ma). On the contrary, the violation

of the rules of purity of gametes, which manifests itself in the phenomenon of absorption, should occur at the level of phenogenetically inactive micronuclei (Mi) and products of their meiosis (i.e. male and female pronuclei). Most likely, the events occurring in the Ma may affect the genes in Mi. In ciliates, phenomena which could be considered as a kind of gametic nuclei predetermination (in particular, for inheritance of serotypes), were described (Sommerville, 1970).

In the light of these data, there was an urgent need to study the expression of serotypes in clones of dilepti, in particular under the influence of temperature and some other factors. It is known that in *Paramecia* and *Tetrahymena*, when they are cultured under standard conditions, the majority of serotypes are relatively stable but some other serotype can be expressed as a result of changing the culture conditions — thus, serotype transformation occurs. Among many agents that can cause the serotype transformation in ciliates, temperature is the best studied factor (see reviews: Beale, 1957; Finger, 1974; Sonneborn, 1975; Preer, 1989). In *D. anser*, change in cultivation temperature from 25 to 17° C resulted in cessation of immobilizing effect of the antiserum raised against the 25-degree ciliates (Uspenskaya, 1990; Uspenskaya and Yudin, 1992). These results suggest that ciliates cultivated at 17° C show some other serotype; serotype transformation has occurred, i.e. replacement of one surface antigen with another took place. Especially demonstrative the serotype transformation due to temperature change was in experiments using simultaneously two ISs against two subclones of #5D clone, one of which was cultured at 17° C, and the other — at 25° C. Each serum immobilized only homologous cells clearly showing change of serotype when ciliates were transferred from 25 to 17° C and vice versa. Acquiring new serotype and loss of the previous one occurred at the same rate, and at 25° C it was faster than at 17° C (Uspenskaya, 1990; Uspenskaya and Yudin, 1992).

Serotype changes that were observed when moving dilepti to different temperature conditions showed complete reversibility when the cells were returned to the initial temperature at which they were originally cultivated. Immunofluorescence analysis showed that the changes observed in these clones did not occur due to the selection of cells with a different serotype, but by means of gradual transformation of all cells in the culture. Hence, the same cells showed one serotype at 17° C and

some other one — when cultured at 25° C. For many serotypes in *Paramecium* and *Tetrahymena*, temperature limits for expression of their i-antigens were determined. For #5D clone, we were able to determine fairly accurately the upper temperature limit for manifestation of one serotype, which we designated as “cold” one, and the lower temperature limit for display of another serotype only, which was called “warm” one. Under prolonged culturing of this clone at intermediate temperatures (23, 21, and 19° C), dilepti showed intermediate levels of immobilization reactions when exposed to both anti-25 IS and anti-17 IS. In other words, within this temperature zone (19–23° C) the ciliates were able to display both “cold” and “warm” serotypes simultaneously (although in various proportions), and this fact also violates the principle of mutual exclusion in the expression of surface antigens (Uspenskaya, 1990; Uspenskaya and Yudin, 1992). In the experiments with *Paramecia*, the temperature transformation was an important auxiliary element in hybridological analysis of serotypes (Beale, 1954). As for *D. anser*, with no segregation among F2 clones it remains unclear whether the serotypes of “parent” clones B and D are controlled by alleles at one locus or by different loci. Situation could be clarified in the experiments on temperature transformation of serotypes in F2 clones. Such temporary “switch-off” of expression of a particular serotype would make it possible to assess the degree of stability of epigenetic changes that have occurred and have been manifested in the absence of segregation and maintaining a “hybrid” phenotype in all F2 clones as well. If the maintenance of these changes directly depends on the expression of serotype, then one would expect that the temperature transformation in F2 clones, i.e. temporary “switch-off” of their “hybrid” phenotype, might lead to changes in the established state of the corresponding epigenetic system and to manifestation of genotypic segregation, which was to take place among the F2 clones.

25 and 26 F2 clones of *D. anser* were used from backcrosses F1 x B and F1 x D, respectively (Table 1). All F2 clones reacted more or less uniformly to both ISs raised against parental clones B and D, i.e. they had a “hybrid” phenotype that persisted during asexual reproduction. In other words, there was no segregation for the analyzed characteristic. These clones were transferred from 25 to 17° C, and serotype transformation was induced. After 2 weeks the clones were returned to 25° C and then, after 7 and 20 days after their return from 17 to 25° C, the

Table 1. Testing of F₂ clones *Dileptus anser* obtained from back-crossing F₁ × #B and F₁ × #D by anti-“parent” immune sera (anti-B IS and anti-D IS) after transfer from 25° C to 17° C for 2 weeks and following cultivation for 1 day at 25° C* (modified from Uspenskaya and Yudin, 2001).

F ₂ clones from F ₁ ×B)	Percentage of cells immobilized by		F ₂ clones from F ₁ ×D	Percentage of cells immobilized by	
	anti-B IS	anti-D IS		anti-B IS	anti-D IS
11-7	0	5	12-7	0	0
13-10	5	0	14-8	0	0
15-2	4	2	16-3	3	1
15-6	7	3	16-5	4	2
17-4	4	4	18-9	1	6
19-1	6	5	20-2	3	3
19-4	3	4	20-3	3	2
19-6	2	0	20-9	2	0
			20-10	4	7

* In each case the proportion of the immobilized cells is defined for 100 cells.

clones were tested for reaction of immobilization. It was found that each clone without any exception responded more or less similarly to both ISs, i.e. manifested joint expression of the parental surface i-antigens (“hybrid” phenotype). This occurred in spite of the fact that the expression of these i-antigens was “switched-off” for two weeks by transfer of the clones to modified temperature conditions (Uspenskaya and Yudin, 2001). Some clones showed significantly unequal response to two test ISs. In some clones, the type of the reaction to both test sera was identical before and after the temporal change of serotype; other clones demonstrated the variable reaction.

In many models proposed to explain the regulation of differential expression of i-antigens in *Paramecium* and *Tetrahymena*, regulatory functions were attributed to i-antigens themselves (Capdeville, 1979; Finger et al., 1995, 1995/1996). A critical review of earlier models of this kind can be found in Doerder (1979). We hypothesized that the lack of segregation for serotypes and co-expression of “parental” antigens in F₂ clones of *D. anser* is a consequence of disruption of the normal regulation of i-antigens expression which took place in the “parental” clones, and looked forward to the restoration of normal regulation after temporary “switch-off” of the jointly manifested i-antigens. However, after a temporary temperature transformation of the serotype, all clones restored the former “hybrid” serotype, i.e. abnormal expression pattern of the corresponding genes has been recovered, which contradicts the assumption of direct participation of i-antigens in the regulation of their own expression.

Thus, in the study of *D. anser* serotypes we observed violations of the principle of mutual exclusion in the expression of i-antigens. In one clone we found prolonged joint expression of two i-antigens at temperatures intermediate between those which induced the complete transformation of one antigenic type to another in this clone. Another failure of the normal mechanism for mutual exclusion of i-antigens was presented with a lack of segregation in F₂ progeny. However, in most cases only one type of surface i-antigen is expressed at a certain time under given conditions, meaning that mutual exclusion operates normally, i.e. control of serotypes in dilepti has an epigenetic component.

Mating types in *Dileptus anser*

Apart from serotypes we investigated another character that is classical for ciliate genetics — mating types (MTs). Since the discovery of mating types in *Paramecium aurelia* by Sonneborn (1937), more and more species of ciliates had been involved in the study of this trait. As a result, a large amount of data on the physiology, biochemistry and molecular biology of sexual processes and mating types in ciliates has been accumulated (see reviews: Tsukii, 1988; Miyake, 1996). MT systems in these organisms, their genetic control and mode of inheritance appeared to be quite diverse (Phadke and Zufall, 2009). There are systems where MTs are directly and unambiguously determined by genes and their alleles. On the contrary, some MT systems were discovered where multipotential MT locus was found to undergo epigenetic differentiation, which resulted in phenotypic realization of only one of

several hereditary genotypic potencies (reviews: Sonneborn, 1977; Tsukii, 1988; Dini and Nyberg, 1993; Nyberg, 1993; Bleyman, 1996; Miyake, 1996). Specific molecular mechanisms of cell determination and differentiation for MT in ciliates were revealed later (see, e.g.: Cervantes et al., 2013; Singh et al., 2014; Vallesi et al., 2014). It was of undoubted importance for us to study the case of *D. anser* in this respect. In particular, the study of MTs in *D. anser* was of interest because dilepti belong to the few ciliates that release specific signaling molecules into the environment — mating pheromones, or gamones that trigger sexual process. Meanwhile, mating type of the organism is determined by the pheromones (1) which a ciliate excretes into the environment, and (2) those excreted by other cells of this species, to which it is able to respond. In other words, differentiation of the mating type in dilepti is associated with the expression of genes that control the production of pheromones and their receptors. The signaling role of gamones, their role in gene expression and relationship with other signaling systems are intensively studied, for instance, in the ciliates *Euplotes* sp. (e.g. Luporini et al., 2005, 2014a, 2014b, and others).

Only three mating types have been found in *D. anser* so far (Tavrovskaya, 1974, 1976). Thus, the MT system in dilepti seems to belong to what is termed “closed MT systems”. These three MTs — MT I, MT II and MT III — are apparently inherited without any changes during vegetative (agamous) reproduction of the ciliates. Individuals differing in their MTs are capable of conjugation (sexual process). After conjugation, two conjugants separate, begin to feed and reproduce by binary fission (agamous, or vegetative reproduction), and form exconjugant clones, two from each pair of conjugants (collectively referred to as synclone). For some time, dilepti of the exconjugant clone are not able to mate with cells of a complementary MT and to engage in the following conjugation; like many other ciliates, they have a period of immaturity. In *D. anser* this period lasts, according to our data, on average about 15 weeks (from 10 to 21 weeks in different clones, i.e., approximately 70–150 cell divisions). After this time span typically there is a short period of partial maturity (adolescence), when a partially mature clone forms pairs with only one of the three clones-testers. Afterwards, the maturity period starts, when the cells of any one of the three MTs can conjugate with the cells of two other, complementary MTs. The maturation period

can be reduced by using micrurgic fragmentation of cells from exconjugant clones (Tavrovskaya, 1981; Uspenskaya and Yudin, 2004).

We have made hybridological analysis of the mating type character in *D. anser*. MT of any clone was determined by crossing the clone with three mature test-clones. In the first series of crosses (Yudin and Afon'kin, 1987), clones #11 (MT I), #20 (MT II), #8, #12 and #28 (MT III) were used. To obtain surely heterotypical pairs, we put together in the same well only one cell from each “parental” clone, removing excess products of preconjugal divisions, or, in some cases, marked cells of one of the clones with Indian ink. After the period of immaturity (which lasted about 80–120 days, with 1–2 divisions per day), MT of the offspring clones was determined by the reaction with the test clones. From the induced selfing-pairs, we have obtained: in clone #11 — only MT I offspring, in clone #20 — only TC II. Offspring from cross #11 × #20 was, with a few exceptions (cytogamy?), TC I, showing synclonal inheritance of the trait. (Recall that synclonal inheritance and Mendelian behavior of MT in the crosses indicates the so-called direct genetic control of the trait — Tsukii, 1988). By mating #20 × #8, #20 × #12, and #20 × #28 we received descendants (including synclones) of only TC II. The backcrossing F1 (#11 × #20) × #20 gave synclones with MT I and MT II. These results suggested that the MT in *D. anser* is controlled by the *mat* locus with three alleles that show serial (hierarchical) dominance ($mat^1 > mat^2 > mat^3$). However, this scheme did not explain the results of hybridization of MT I × MT III (#11 × #8, #11 × #12 and #11 × #28), from which, in addition to synclones MT I, synclones MT II and synclones consisting of a clone of MT I and MT II were received. The nature of these exceptions has not yet been analyzed.

Interestingly, similar results were described in the study of inheritance and genetic determination of MTs in the ciliate *Tetrahymena pigmentosa* (Orias, 1963; Simon, 1980). In both cases, there are three MTs that are inherited during conjugation synclonally and in such a way as if they were controlled by a single locus with three alleles that show serial dominance. At the same time, in the cited publications on *T. pigmentosa* some unexplained deviations from this scheme were revealed that were expressed, in particular, in excess segregants of MT II in the progeny from crosses and in the presence of a small fraction of clones-selfers (Orias, 1963).

In the second series (Yudin and Uspenskaya,

2006), 20 crosses were made, and two kinds of results were received. In a number of crosses, there were observed: 1) synclonal inheritance of the character MT; 2) dominance of MT I over MT II and MT III, and MT II – over MT III; 3) segregation for MT that did not differ significantly from the expected proportions. In other words, more or less obvious “Mendelian” behavior of a monofactorial trait was observed, and the whole picture was in accord with the hypothesis of genetic control of the MTs in *D. anser* previously suggested by Yudin and Afon'kin (1987). However, in a number of crosses of *D. anser* we also observed more or less significant deviations from the suggested scheme. In some crosses, exconjugant clones with abnormal behavior with respect to their MT (sometimes in addition to “normal” exconjugant clones) were observed. Abnormal features of these clones were: (a) a delay in maturation, as compared with “normal” clones, (b) temporary return from the mature state to immature or adolescent one (i.e., instability of maturity state – like it was observed by Dini and Nyberg (1994) in *Euplotes crassus*), (c) changes of MT at the early period of maturity, repeated rather frequently, (d) emergence of clones unexpected in a given cross according to the above scheme of genetic control of MT, (e) non-Mendelian proportions of different MTs including the total absence of some expected classes of segregants (Table 2). Out of 142 tested exconjugant clones obtained in 8 different crossings, the change in MT (single or double) was noted only in 40 clones (28%). In 30 cases out of 45, we managed to register that the change in MT involved a state of immaturity or partial maturity. Changes of MT occurred in all possible directions, although with different probability. So, from 45 observed changes in MT 25 changes were to MT III, and only 8 – to MT I. There is an impression that the change of MT was likely due to the most recessive MT (Uspenskaya and Yudin, 2003; Yudin and Uspenskaya, 2006).

Meanwhile, it remained unknown whether any differences in the behavior of “normal” and “abnormal” exconjugant clones were due to genetic differences between them. It was also unclear how correlated were the behavior of sister clones in a synclone, deviations from Mendelian ratios of different MTs in exconjugant offspring, and instability of MT in young exconjugant clones. The synchrony and direction of changes in MT in all individuals of a culture in unstable exconjugant clones and the lack of selfing in them still was ambiguous. It also remained unknown whether

it was possible to affect the change of MT during maturation of unstable clones, and if so, what were the probable impact factors. It also remained unclear how long such unstable condition retains in young exconjugant clones. It could not be excluded that these clones, ending the process of differentiation of their macronuclei, lose all their genetic potentialities for MT except one, become stabilized and then steadily inherit their MTs. In this case, Ma of young exconjugant clones and Ma of old mature clones had to differ genetically with respect to MT character, and mature clones most likely were not able to become destabilized and modify their MT in a number of vegetative generations. On the contrary, if the determination of the trait has an epigenetic nature, then mature clones can store all the genetic potentialities for three MTs in their macronuclei and are capable of changing these characteristics.

To test this suggestion, an attempt was made to cause changes of MT in old laboratory clones of *D. anser* with the help of some external influences. As an inducing agent, actinomycin D (AMD) was selected. Previously, it was found that AMD induced inherited instability of a number of the cell nucleus–controlled hereditary traits in another protozoan species, the amoebae *Amoeba proteus*, which according to many criteria had epigenetic nature (Kalinina, 1968; Olenov, 1970; Yudin 1979, 1981). It is known that this antibiotic is not mutagenic in a conventional sense (see review: Koba and Konopa, 2005). It was previously noted that mature *Dileptus* clones that had been isolated from nature and subsequently cultivated in the laboratory mostly preserved their mating type over many months and even a year of observations. In our experiments, working concentration of 15 µg/ml was chosen, at which 75% of AMD-treated cells survived as compared to 100% survival in the control (see detailed description of the experiments in: Uspenskaya and Yudin, 1996). Three laboratory clones were used: #3 (MT I), #13 (MT II) and #153 (MT III). Dilepti were incubated in the antibiotic solution for 3 days, and then washed, isolated individually, and the clones were further cultured traditionally. 20 subclones were grown for each clone, which were then weekly tested for their MT for four months. During the testing a subclone, its immaturity (lack of mating reaction with any of three tester clones), or the partial maturity (mating reaction with only one tester clone) were also registered. Basing on the results of these experiments, we can conclude that AMD destabilized TC of the

Table 2. Testing of mating type in just matured exconjugant clones of *Dileptus anser* (several examples, from Yudin and Uspenskaya, 2007).

Clone	MT of the clone (Roman numerals) and period of time after conjugation (in brackets, months)
Progeny from the cross # B (MT I) x # D (MT III); a total of 7 clones obtained from this cross were tested	
5-14	II (5-6) → IM (9-10) → I (14-15) → I (19-20)
7-3	I (5-6) → IM (9-10) → II (14-15) → II (19-20)
9-7	III (9-11) → (14-15) → II (19-20)
10-21	II (5-6) → IM (9-10) → III (14-15) → III (19-20)
Progeny from the cross F ₁ (MT II) x # B (MT I); a total of 5 clones obtained from this cross were tested	
13-10	I (3-4) → IM (5-6) → IM (9-10) → AD (14-15) → II (19-20)
Progeny from the cross F ₁ (MT II) x # D (MT III); a total of 5 clones obtained from this cross were tested	
14-2	II (3-4) → IM (5-6) → AD (9-10) → III (14-15) → II (19-20)
18-3	III (9-10) → II (14-15) → I (19-20)
20-2	II (9-10) → AD (14-15) → I (19-20)

Notes: IM – immaturity (the clone does not mate with any one of three tester clones); AD – adolescence (clone mates with only one of three tester clones); F₁ – progeny from the cross # B x # D.

mature, long maintained in the laboratory clones of *D. anser*. After treatment with sublethal doses of the antibiotic, there was a more or less prolonged state of immaturity or partial maturity of the treated clones. In addition, their MTs could change to one of two other possible MTs for a more or less prolonged period of time. For example, MT in the clone #3 changed from MT I to MT II and backward, in the clone #13 – from MT II to MT I and back, and in the clone #153 – from MT III to MT I and (in one case) to MT II and backward (Table 3). It should be emphasized that the temporary state of immaturity, the condition of transient expression of a particular MT, and the very state of instability in the destabilized clones were inherited over dozens of vegetative generations (cell heredity). In general, the behavior of the AMD-treated clones was similar to the behavior of many “young”, just matured exconjugant clones that also exhibited instability of their MTs (Uspenskaya and Yudin, 2003; Yudin and Uspenskaya, 2006, and others).

In our opinion, the results obtained in this experiment prove that in *D. anser* that stably reproduces one MT during the vegetative propagation, macronuclei retain genetic potentialities for developing two other MTs known in this species. These latent potentialities can be activated in certain conditions or after certain treatments. The mechanism of this activation remains unknown. It is also important to mention that in two very different protozoan species — amoebae and ciliates — AMD causes epigenetic changes with high frequency. This allows suggesting that AMD is a specific inducer of

epigenetic changes, or an “epimutagen” (Yudin and Uspenskaya, 2009). As regards the possible molecular mechanisms of the epimutagenic activity of AMD, this question is obviously linked to the problem of molecular mechanisms of epigenetic differentiation and transdifferentiation of MTs in *D. anser*, which are still unresolved. The fact that long cultivated clones of *D. anser*, which permanently express one of three possible MTs, under certain conditions are able to express other MTs, indicates that in maturing dilepti differentiation of their macronuclei for MT cannot occur by mechanism of DNA deletions and splicing in a complex *mat* locus (Orias, 1981; Cervantes et al., 2013). In this case, whatever are the specific molecular mechanisms of epigenetic changes and their inheritance, one of the possibilities is a control of MTs in *Dileptus* by a complex locus encoding potency for all three MTs, occurring in this species. This locus is inherited as a whole and retains its complexity in Ma. However, it can undergo epigenetic differentiation according to the principle of mutual exclusion, in which only one of the three genetic potentialities encoded in its genome activates, and the other two are inactive. As a result of this differentiation, there might be three epialleles of this complex locus that determine MT I, MT II and MT III. The degree of stability of such differentiation may vary inexplicably. If the differentiation is stable, the epialleles functionally behave like normal alleles of the genetic locus, as if they arose by mutations: there is the usual Mendelian behavior of MT with serial dominance, monofactorial segregation, etc.

Table 3. Maturation of *Dileptus anser* clone 153 (MT III) after the treatment by actinomycin D (15 µ/ml, 3 days) (from Yudin and Uspenskaya, 2009).

Subclones of clone 153	Mating type of subclones at consecutive testing (weeks after incubation)							
	1–2	3–4	5–6	7–8	9–10	11–12	13–14	More than 15
153-1	III	–	–	I	I	dead		
153-2	III	–	III	–	II	II	II	II
153-3	III	–	–	–	I	I	I	I
153-4	–	–	–	III	III	III	III	III
153-5	III	III	III	III	III	III	III	III
153-6	I	–	–	I	III	III	I	I
153-7	III	–	–	–	–	II	–	II
153-8	III	III	–	–	–	dead	–	–
153-9	–	–	–	II	II	II	II	II
153-10	–	–	–	–	–	dead		
153-11	III	–	–	–	dead			
153-12	III	–	–	III	III	III	III	III
153-13	–	–	I	I	I	I	–	dead
153-14	III	–	II	II	–	–	dead	–
153-15	–	–	III	II	–	–	II	–
153-16	III	III	III	III	III	–	–	dead
153-17	–	–	II	–	–	I	I	I
153-18	III	III	III	III	III	III	III	III
153-19	–	–	I	I	–	–	I	I
153-20	–	II	–	II	–	II	II	II

Notes: The dash (–) indicates that the subclone does not react with any of the three test clones (immaturity state). Bold font indicates the cases of MT manifestation that are not characteristic of the initial clone # 153.

If epigenetic differentiation of the locus is more or less unstable, then the switching of activity from one potency to another is possible – a kind of epimutation. This may disrupt normal Mendelian pattern: relations of dominance – recessivity between epialleles, character segregation, etc. And, in addition, instability of a MT expressed in maturing exconjugant clones can occur (Yudin and Uspenskaya, 2007). In general, genetic control of mating types in the ciliate *D. anser* appears to be rather complicated and needs further investigation.

In many respects our experiments of inheritance of serotypes and mating types in the ciliate *Dileptus anser* do not meet the strict requirements of genetic analysis. Unfortunately, this is inevitable when working with a species that has not yet become a widely known laboratory model. Our attempt to reproduce the findings obtained earlier with other organisms was justified. We observed new, previously undescribed phenomena and features in the breeding system of *D. anser* (namely, the simplest possible system of multiple mating types, serial, or hierarchic; full dominance of the alleles in *mat* locus; unusually small size of mating pheromones, etc.). We found

deviations from the principle of mutual exclusion in serotype expression of this species. Non-Mendelian behavior of serotypes and mating types was registered as well. Thus, we proved *D. anser* to be a new prospective and convenient model for comparative research in genetics and epigenetics of ciliates. One of the possible prospects is testing our hypothesis about the epigenetic nature of the determination of mating types in this ciliate and analyzing its molecular mechanisms. As for the mechanisms of internuclear interactions in ciliates, recently there appeared data on the information role of small RNAs, mainly in the internuclear interactions, which control very complex and very precise rearrangements of the germline genome, occurring in the developing Ma after the conjugation. Among the latter, the model of scanning RNAs is the most widely discussed (Meyer and Chalker, 2007; Duharcourt et al., 2009; Nowacki and Landweber, 2009). Further studies on pheromones in *D. anser* will contribute significantly to the investigation of intercellular recognition in lower eukaryotes and the related issues.

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