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New results on the genetics of mating types in *Paramecium bursaria**

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1. INTRODUCTION

The discovery by Jennings (1938) of multiple mating types in *Paramecium bursaria* formed the basis for genetic investigations of this ciliate. Four mating types are known for syngen 1; when animals of complementary mating types are mixed, conjugant pairs form in large numbers and new sexual generations are inaugurated. The initial problem posed by Jennings' work, that of the inheritance and determination of mating type, was resolved in part when it was shown (Siegel & Larison, 1960) that pairs of alleles at two independently assorting loci may be combined in four ways, each determining a specific mating type. Mating-type I is brought about by dominant genes at both loci, mating-type III is determined by the double recessive, while types II and IV are determined by recessive alleles at one or the other of the two loci in combination with at least one dominant gene at the other locus. The experiments to be reported here provide exhaustive tests of this two-locus hypothesis in that they extend it to new strains collected from nature and to clones synthesized in crosses. Most importantly, they suggest adequate explanations for the results of certain crosses, occurring within a single line of descent, which seemed at first to be entirely exceptional. Finally, the events of fertilization and nuclear reorganization in this species, previously based on cytological description, have been analysed with the aid of the two marker loci concerned with mating types.

2. MATERIAL AND METHODS

The twelve strains of *P. bursaria* used in the present studies are listed in Table 1. Each consists of the vegetative progeny of a single cell collected from the indicated natural source. Each of the strains is pure for the indicated mating type. In the past, considerable confusion has arisen due to the fact that letter symbols have been used to designate the four mating types, the genes controlling these mating types, and the 'mating-type substances' postulated by Metz (1954) as the physical basis for mating specificity. Since Roman numerals have been used to designate mating types in other ciliates it is proposed that this system be extended to

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P. bursaria. The mating types of syngen 1 formerly lettered A, B, C and D are now assigned the numerals I, II, III and IV respectively. In keeping with past publications the dominant and recessive alleles at one locus are designated by letters *A* and *a*, while those at the second locus are *B* and *b*.

The cells were cultured in baked-lettuce infusions (see Sonneborn, 1950) inoculated with the bacterium *Aerobacter cloacae*, which served as the food organism. Mass cultures were maintained in test tubes at 24–26° C. and enough fresh medium was provided to permit about three fissions per week. They received either indirect sunlight or artificial illumination of 100 foot-candles supplied by daylight fluorescent lamps for 10 hours each day; otherwise the cultures were in total darkness. The cells reached a peak of mating reactivity at noon and crosses were initiated at that time.

Table 1. *The strains, their natural sources and mating types*

Strain number	Source	Mating type	Strain designation
3	Malibu Lake, California	IV	3-IV
8	Malibu Lake, California	I	8-I
25	Malibu Lake, California	II	25-II
32	Malibu Lake, California	III	32-III
34	Fish Canyon, California	III	34-III
41	Lake Fulmer, California	II	41-II
42	Marymount Pond, California	I	42-I
45	Cottonwood Stream, California	I	45-I
395	Susquehanna River, New York	I	395-I
442	Hemet Lake, California	I	442-I
2681	St. Mary's River, Florida	II	2681-II
JP-55	Sapporo, Japan	III	JP-55-III

It was important to select for genetic study only those pairs of conjugants which reciprocally exchanged gametic nuclei and then passed through nuclear reorganization. At 25–27° C. conjugation requires about 20 hours, and reorganization lasts an additional 36 hours; during these periods the cells will not multiply. If mates separated precociously or if the exconjugant cells underwent fission before 36 hours, they were excluded from the analysis.

Exconjugant clones were maintained in continual darkness at 27° C. in serial isolations permitting maximal fission rates; under these conditions most clones required 50 to 100 fissions (two to four weeks) to reach sexual maturity. The isolation lines were transferred at this point to test tubes, and allowed to form mass cultures. These could be conveniently tested for mating type and used in further crosses. Mating types were determined on the basis of tests with cells of the four standard mating types. The unknown clone was classified as a particular type if it failed to mate with the standard of that type but did mate with cells of the remaining three types.

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3. RESULTS

(i) *Cytogenetics*

Hamburger's (1904) cytological studies of the events of conjugation in *P. bursaria* have been extended by Chen in a series of brilliant and detailed papers (1940*a*; 1940*b*; 1940*c*; 1946*a*; 1946*b*; 1951*a*; 1951*b*) which describe both normal and abnormal (or lethal) matings. Following the union of cells, the single micronucleus in each mate passes through two meiotic divisions; one product of each division disintegrates and so a single reduced nucleus survives. This undergoes a mitotic division to produce the male and female gamete nuclei. The male gamete nuclei are reciprocally exchanged between mates and then fuse with the stationary female gamete nuclei to form a syncaryon in each conjugant. Each syncaryon divides once and one product disintegrates; the surviving nucleus then divides twice and its four products differentiate into two macronuclei and two micronuclei. At about this time the conjugants separate and at the first cell division the nuclei are segregated to the fission products so that the normal nuclear condition of one micro- and one macronucleus per cell is restored. The prezygotic macronucleus appears to waste away.

These cytological observations suggest that (1) the third prezygotic micronuclear division is mitotic, (2) the mates reciprocally exchange gametic nuclei and (3) the three postzygotic divisions of the syncaryon, whereby micro- and macronuclei are formed, are mitotic. In agreement with these three points, each pair of conjugants should produce a pair of exconjugant clones with identical phenotypes (determined by macronuclear genes) and identical micronuclear genotypes. Since at least 97% of all matings give pairs of exconjugant clones which are phenotypically alike (see Siegel & Larison, 1960), it may be inferred that macronuclei which originate in a common reciprocal fertilization are regularly isogenic. In order to test the conclusion that all progeny of any given pair will have identical micronuclear genotypes, the four caryonides from thirteen pairs were isolated, grown to sexual maturity and then test-crossed to the double recessive, mating-type III. The genotype of the micronuclei of each caryonide so crossed was revealed by the distribution of mating types among the test-cross progeny. For each pair studied, the four caryonides were found to have isogenic micronuclei with respect to the two loci (or chromosomes) tested.

Various abnormal cytogenetic events have been observed in related species of *Paramecium*. How frequent are (1) the failure of reciprocal gamete exchange (Wichtermann, 1939) and (2) the 'parthenogenic' development of the female gamete nuclei (Sonneborn, 1954) in *P. bursaria*? Each pair from the cross of mating-types II (*aaBB*) and IV (*AAbb*) should give, normally, progeny of mating-type I (*AaBb*); the appearance of exceptional progeny of mating-type II or IV might indicate the existence of the kinds of exceptional processes noted above. Since no exceptional progeny appeared among the descendants of 103 pairs isolated from this cross, it is safe to assume that such abnormalities are extremely rare in the present material.

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(ii) *Crosses among the standard strains and their sexual progeny*

In our first study on the genetics of mating types (Siegel & Larison, 1960), the relationships between genotype and phenotype shown in Table 2 were established. The three standard strains with genotypes *AaBb*, *aaBb* and *Aabb* were crossed to produce new genotypes which were identified by test-crosses to the double recessive.

Table 2. *The four mating types and their genotypic control*

Mating type	Genotypes found
I	*AABb*, *AaBB*, *AaBb*
II	*aaBB*, *aaBb*
III	*aabb*
IV	*AAbb*, *Aabb*

The eight genotypes shown in Table 2 have now been crossed in all twenty-three possible pairwise combinations of complementary mating types, their progeny isolated and scored for mating types. The ratios of F_1 mating types were found to support in detail the genotypes originally assigned to the parental clones; moreover the absence of types II, III and IV among the progeny from crosses of *AABb* × *aaBB*, *AaBB* × *AAbb* and *aaBB* × *AAbb* provides new qualitative support for the two-gene hypothesis.

Exceptional results have been noted among clones in one line of descent; these clones and their progeny will be discussed in a later section.

(iii) *Generalization of the two-gene hypothesis to new strains and to clones studied by Jennings*

The two-gene hypothesis was based initially on the analysis of only four strains and their sexual progeny; hence it seemed important to explore the generality of the hypothesis by the analysis of new material and reanalysis of Jennings' results. Eight new strains, each derived from a single animal which had been isolated from a new natural source (see strains 34 through JP-55 listed in Table 1) were tested for mating type and genotype. None proved to be exceptions to the hypothesis (see Table 3). Secondly, Jennings (1942) has recorded the mating types for the progeny of many crosses; genotypes which are consistent with the hypothesis can be deduced for eighteen of his parental clones. Table 3 summarizes these results.

Four clones intensively studied by Jennings may be exceptions to the hypothesis and have been omitted from Table 3. The data available for these do not permit a full and satisfactory interpretation; on the other hand, exceptions which were noted in the present material have been studied and will be described next.

(iv) *The analysis of certain exceptional results*

Jennings' crosses and crosses in this laboratory have produced from time to time certain unexpected progenies. Unfortunately, Jennings' clones are no longer available for study and, as he and others appreciated, the data did not exclude a number

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Table 3. *The relationship of genotype and mating type for eight newly collected strains and eighteen clones studied by Jennings*

Strain or clone number	Studied by	Mating type	Genotype
42	RWS	I	*AaBb*
45	RWS	I	*AaBB*
395	RWS	I	*AaBb*
442	RWS	I	*AaBB*
2	HSJ	I	*AaBb*
9	HSJ	I	*AaBb*
33b	HSJ	I	*AaBb*
41	RWS	II	*aaBb*
2681	RWS	II	*aaBb*
5	HSJ	II	*aaBb*
10	HSJ	II	*aaBB*
27	HSJ	II	*aaBb*
35a	HSJ	II	*aaBb*
44	HSJ	II	*aaBb*
34	RWS	III	*aabb*
JP-55	RWS	III	*aabb*
1	HSJ	III	*aabb*
12	HSJ	III	*aabb*
21	HSJ	III	*aabb*
23	HSJ	III	*aabb*
36	HSJ	III	*aabb*
*8 (HSJ)	HSJ	IV	*Aabb*
11	HSJ	IV	*Aabb*
24	HSJ	IV	*Aabb*
32b	HSJ	IV	*AAbb*
39	HSJ	IV	*Aabb*

* This is clone 8 from *Jennings'* collection; not to be confused with our strain 8-I.

of alternative explanations (see Sonneborn, 1957). It is most significant that all of the six exceptional clones found among our crosses occurred in a single line of descent; the origin of these exceptional clones is shown in Fig. 1.

21-IV attracted attention because the remaining three sister caryonides in pair 21 from the cross 8-I × 32-III (*AaBb* × *aabb*) were type I. A breeding analysis of 21-IV established its *micro*nuclear genotype as *AaBb* (see Table 4), consistent with that for the other caryonides of pair 21. Since the phenotype of a cell is dependent solely upon *macro*nuclear genes, the *macro*nuclear genotype for 21-IV is, by inference, *Aabb*; if so, the dominant allele *B* inherited from the parent 8-I must have been lost or in some way altered during the development of the *macro*nucleus—but not the *micro*nucleus—of this caryonide.

The genotype of clone 8-II was established as *aaBB* on the basis of test-crosses

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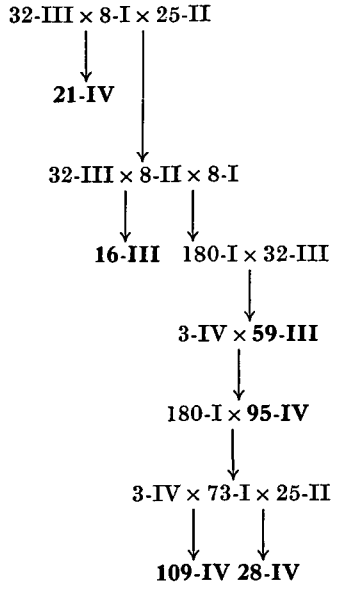

Fig. 1. The origin of the six exceptional clones; exceptional clones are in bold type.

to the double recessive (32-III); of twenty-nine test-cross clones formed, twenty-eight were type II and one was type III. New crosses of the exceptional clone, 16-III, revealed a *micro*nuclear genotype *aaBb* (see Table 4), in agreement with that expected for the cross *aaBB* × *aabb*. On the other hand, the expression of mating-type III requires a *macro*nuclear genotype *aabb*. As in the case of 21-IV, the *macro*nuclear genotype for this clone appears to differ from its *micro*nuclear genotype and again the change involves the *B*-locus.

Table 4. *Crosses which establish the genotypes of four exceptional clones*

Exceptional clone	Crossed to		F_1 progeny formed I	II	III	IV	χ^2	Genotype of exceptional clone: Micronucleus	Macronucleus
21-IV	181-I	*AaBB*	29	4	0	0	2·27	*AaBb*	*Aabb*
	292-I	*AABb*	23	0	0	10	0·25		
	25-II	*aaBb*	11	15	1	9	7·85*		
16-III	25-II	*aaBb*	0	10	3	0	0·03	*aaBb*	*aabb*
	3-IV	*Aabb*	4	5	2	5	1·50		
59-III	8-I	*AaBb*	7	8	1	2	0·96	*aaBb*	*aabb*
	25-II	*aaBb*	0	97	43	0	2·14		
	3-IV	*Aabb*	5	6	9	12	3·88		
95-IV	8-I	*AaBb*	73	14	4	20	5·29	*AaBb*	*Aabb*
	25-II	*aaBb*	49	32	17	13	4·34		
	32-III	*aabb*	7	5	1	6	4·07		

* Chi-square value significant at the 0·05 level.

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Clone 180-I (*AaBB*) was formed in a backcross of 8-II (*aaBB*) to parental strain 8-I (*AaBb*). Of 105 F_1 clones from the cross 180-I × 32-III (*AaBB* × *aabb*), 103 were types I and II in the expected 1:1 ratio. Two exceptional mating-type III clones were found; one of these, 59-III, has been studied but the other died off soon after it was discovered. The data (see Table 4) establish the *micro*nuclear genotype *aaBb* for this clone and, since mating-type III is expressed, the inferred *macro*nuclear genotype is *aabb*. Again the dominant allele *B* appears to be missing or altered in the *macro*nucleus.

Among the sixteen sexual progeny of 59-III × 3-IV (*aaBb* × *Aabb*), only clone 95-IV was exceptional. The cells of this clone were shown to have the *micro*nuclear genotype *AaBb* (see Table 4) but since they express type IV, they presumably carry the *macro*nuclear genotype *Aabb*. Clone 73-I (*AABB*) is a direct descendant of 95-IV and it in turn produced two instances of exceptional progeny in crosses to strains 3-IV (*Aabb*) and 25-II (*aaBb*) as shown in Table 5. Both exceptions expressed mating-type IV and crosses to the double recessive showed that both have the *micro*nuclear genotype *Aabb*. These two clones are similar to the four exceptionals discussed above in that they can be interpreted as arising from nuclei in which the dominant allele *B* has become altered or lost. But whereas the exceptional event in clones 21-IV, 16-III, 59-III and 95-IV is restricted to the *macro*nucleus, 28-IV and 109-IV carry exceptional *micro*nuclei as well. The simplest interpretation of these two clones is that instability of the *B*-locus occurred during gametogenesis or early in the development of the syncaryon so that both the *micro*- and *macro*nuclei of these clones were exceptional.

(v) *The new genotype* AABB

As a final test of the two-gene hypothesis, it was predicted that cells carrying the double dominant genotype *AABB* will be mating-type I. This genotype cannot ordinarily be formed because the union of two gametic nuclei with the genotype *AB* would require the conjugation of two cells of mating-type I. The discovery of clones whose *micro*nuclear and *macro*nuclear genotypes differed offered a solution to the problem. For example, clone 95-IV which forms gametes *AB* can be crossed to normal clones of mating-type I which also form *AB* gametes. The analysis of such crosses is reported below.

The cross 180-I × 95-IV produced forty-three clones of type I, twelve of type II and none of types III and IV in agreement with the 3:1 ratio expected for an *AaBB* × *AaBb* mating. In addition, the cross 181-I × 95-IV (*AABb* × *AaBb*) produced a total of thirteen type I to four type IV progeny also in agreement with the predicted 3:1 ratio. From these two crosses sixteen F_1 clones of mating-type I were test-crossed to the double recessive in order to discover which of them, if any, produced only progeny of type I; of these, clones 38-I and 73-I formed only type I among their test-cross progeny, and hence must be genotypically *AABB*. A summary of these and other crosses which served to establish the double dominant genotype for clones 38-I and 73-I is presented in Table 5. The two exceptional clones found among the progeny of 73-I have been described in the preceding section.

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Table 5. *The results of crosses which establish the genotype* AABB *for clones* 38-I *and* 73-I

			F_1 progeny formed			
	Crossed to		I	II	III	IV
38-I	25-II	*aaBb*	49	0	0	0
	32-III	*aabb*	20	0	0	0
	3-IV	*Aabb*	31	0	0	0
73-I	25-II	*aaBb*	21	0	0	1
	32-III	*aabb*	19	0	0	0
	3-IV	*Aabb*	24	0	0	1

4. DISCUSSION

The results reported here, together with those from earlier studies, provide a firm factual basis for the interpretation of future genetic analyses of *P.* bursaria. The much debated and important question of whether or not conjugants regularly exchange massive amounts of cytoplasm has been investigated (Siegel, 1960). It was found that only under special conditions, such as treatment of conjugants with homologous antiserum (Harrison & Fowler, 1945), was cytoplasmic exchange a regular and frequent feature of mating.

A possible pitfall for genetic studies in *P. bursaria* was removed when it was established that as a rule all conjugations are 'legitimate' (Larison & Siegel, 1961). That is to say, in every case where two clones consisting of cells of complementary mating types are mixed together, the pairs which form consist of one individual from one clone and one from the other. An exceptional mutant clone which could be induced to form pairs *inter se* following contact with cells of a complementary mating type was not included in the present investigation.

Uniparental sexual reorganization, or autogamy, has not been reported in the literature on the cytology of *P. bursaria*. Studies with *P. aurelia* where autogamy may occur in all clones show that during this process the nuclear apparatus is replaced and that homozygous individuals are formed. Since mating types in *P. bursaria* are genically determined, autogamy in a singly or doubly heterozygous clone should produce cells of mating types complementary to that of the parental clone, leading to intraclonal conjugations. Many such heterozygous clones have been under observation for the past four years and none have formed cells of new mating types nor shown evidence of self conjugation. It must be concluded that autogamy is extremely rare or non-existent in this species.

Finally, the data indicate that the most common cytogenetic abnormalities observed in related species must be very rare in *P. bursaria*. When one selects pairs which have remained joined for the normal period required for conjugation and selects exconjugant clones which show the normal fission delay following their formation, then aberrant types of fertilization and nuclear reorganization are avoided.

Clones expressing an unexpected phenotype were recovered in six crosses, all

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within a single line of descent. The six exceptional clones are similar in one important respect; all can be explained by a mutational event at the *B*-locus so that the gene *B* is expressed as its recessive allele, *b*. As an alternative, these clones might have lost all or a part of the chromosome which carries the *B*-locus. In four of the exceptions, the *micro*nuclear genotype was found to be in agreement with that expected for the cross; for example, $aaBB \times aabb \rightarrow aaBb$. But the *macro*nuclear genotype in each case, that is, the genotype deduced from the expressed phenotype, was inconsistent with that expected for the cross. If the genotypes of the nuclei differ as proposed here, then it may be concluded that the apparent change $B \rightarrow b$ took place sometime following the origin of the *macro*nucleus from the syncaryon. In the remaining two exceptional lines, the mutation of *B* (or its loss) appeared in the *macro*- and *micro*nuclei of the clone, suggesting that the event occurred before the differentiation of the nuclei or during gametogenesis.

How frequently is the allele *B* altered or lost, and is the change reversible? Six exceptions were detected among a total of 230 progeny clones from crosses in this line of descent, or 2.6% of the clones were aberrant. Parenthetically, Jennings, and later Siegel and Larison, found that 2–3% of conjugant pairs gave clones of dissimilar mating types. Considering the polyploid or polygenomic nature of the *macro*nucleus this would be a minimal estimate for the 'mutation' rate. There is no evidence which might suggest the reverse change of *b* to *B* occurring in either these exceptional clones or in other clones, and such changes might well have been detected as exceptions to the rule of the clonal constancy of mating type. Nor has instability affecting the *A*-locus been found.

The recovery of clones with the genotype *AABB* and the fact that they express mating-type I confirm the two-locus hypothesis for mating-type determination in syngen 1 of *P. bursaria*. Cells belonging to these clones gave no indication of a mating reaction with doubly heterozygous cells of type I, so one must conclude that the alleles *A* and *B* are completely dominant. In this connection it was also observed that the intensity of the mating reaction is the same for cells of the constitution *AABB* and *AaBb*.

Two apparently diverse mechanisms of mating-type control have been reported for the ciliates. The four mating types of *P. bursaria*, syngen 1 are apparently directly controlled by genes at two independently assorting loci. Multiple mating types in certain other ciliates, namely *Euplotes patella* (Kimball, 1942), *E. vannus* (Heckmann, 1961) and *Tetrahymena pyriformis*, syngen 8 (Orias, 1959) are determined by specific combinations of single multiple-allelic series. On the contrary, the seven mating types in *T. pyriformis* (syngen 1) do not appear to be related to specific genotypes; the *macro*nuclei formed by exconjugant cells are differentiated soon after their origin so that but one of several potential phenotypes is eventually expressed. In this system, as in *P. aurelia* (see Sonneborn, 1947), the genotype has been shown to control only the spectrum of possible types (Nanney, 1959) although by analogy with the first group of ciliates, specific mating-type determining loci may exist. If so, then the difference between these two groups of ciliates may not be so radical. Another finding reported by Jennings (1941) may eventually turn

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out to strengthen the idea of the basic unity of these two kinds of mating-type determination; it is clear that while intraclonal mating-type differentiation—or the fact that isogenic cells may express complementary types—is the rule in *P. aurelia* and *T. pyriformis*, syngen 1, it may also occur rarely in *P. bursaria*.

Jennings analysed in detail these exceptions to the rule of clonal constancy of mating type and his observations are particularly important because they are not easily explained by the two-gene hypothesis. Two facts are immediately striking: first, each unstable clone produces just two types, the two types formed being characteristic of that clone. For example, some clones produce types I and II, others types III and IV and so forth; second, the changes in mating type are reversible. If cells of type IV are formed by a clone initially pure for type I, these may again form progeny of type I.

Jennings suggested that instances of mating-type change might be the result of nuclear reorganization (autogamy); but this interpretation is virtually ruled out by the lack of either genetic or cytological evidence for this process in *P. bursaria*. Moreover, if mating types are under genic control, autogamy could not produce reversible changes nor would it explain why just one new type could be formed by all clones of type I. The results could be explained in terms of the kind of mutational events described here, but the major difficulty for such a view is the fact that three of Jennings' eighteen exceptional clones formed types II and IV necessitating the unlikely assumption of instability for both loci.

We have not yet uncovered any exceptions to the rule that each clone consists of cells of a single mating type. Intraclonal mating-type instability was sought in experiments in which a series of isolates were derived from forty-seven different clones and these tested for mating type; mating-type variability was not found in these intensively studied clones nor were instances of intraclonal conjugation (one criterion for the differentiation of new mating types within a clone) observed in the present material. Until the phenomenon is rediscovered and studied in view of newer information, it remains as the most significant skeleton in our closet.

5. SUMMARY

1. The four mating-types I, II, III, IV known for *P. bursaria*, syngen 1 are determined by specific combinations of dominant and recessive alleles at two unlinked loci. Mating-type I is formed by cells with the genotypes *AABB*, *AABb*, *AaBB* and *AaBb*; type II is controlled by the genotypes *aaBB* and *aaBb*; the double recessive, *aabb* is type III, genotypes *AAbb* and *Aabb* bring about type IV.

2. Clones which are exceptions to these rules for mating-type determination occur in low frequency (2–3%) in one line of descent. All can be explained by assuming either a mutation of dominant gene *B* to its recessive allele *b* or to a loss of the *B* locus. The data suggest that in four clones, the *macro*nuclear but not the *micro*-nuclear genotype is affected; in two clones both nuclei are aberrant.

3. The cytogenetic events of conjugation were verified by the use of the new genetic markers. No evidence for uniparental nuclear reorganization, autogamy, was found.

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