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Maintenance of heterozygosity at the *mt* locus after autogamy in *Euplotes minuta* (Ciliata Hypotrichida)*

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1. INTRODUCTION

As is well known, sexual reproduction in ciliates is brought about either by conjugation or autogamy; the former is by far the more widespread sexual process, whereas the latter has so far been found to occur in very few species (Corliss, 1965; Siegel & Heckmann, 1966; Nobili, 1966*b*). Conjugation involves union of the animals in pairs. In some species of the genus *Euplotes*, cytologically analysed during conjugation (Turner, 1930; Katashima, 1960; Heckmann, 1963–64; Nobili, 1966*a*), it has been found that the micronucleus of each conjugant first of all divides mitotically; then, the two resulting nuclei pass through meiosis, giving rise to eight haploid nuclei. While six of these nuclei degenerate, the remaining two divide once more. Two of the four products disintegrate and the other two become the functional pronuclei. One pronucleus of each conjugant migrates into the partner with whose stationary pronucleus it unites, to form a diploid fertilization nucleus, or synkaryon. The normal nuclear apparatus of the species is then reconstituted by the differentiation of a new macronucleus and a new micronucleus from the products of two successive mitoses of the synkaryon. Autogamy appears to involve the same nuclear processes as conjugation, except that cross-fertilization is impossible because the animals are not united in pairs. Thus, the synkaryon, or fertilization nucleus, is formed by the union of two pronuclei from the same individual.

Cytological details of autogamy and its genetic consequences have been thoroughly analysed in *Paramecium aurelia* (Sonneborn, 1947). When heterozygotes of *P. aurelia* pass through autogamy, all the new clones are homozygous. This fact, together with cytological evidence, proves that the two pronuclei uniting to form the synkaryon are the mitotic products of a single haploid nucleus.

The data presented by Siegel & Heckmann (1966) suggest that some recombination may take place after autogamy in heterozygous *Euplotes minuta* specimens; however, as they pointed out, both cytological and genetic details of the process are still obscure. The present paper will mainly deal with the genetic aspect of autogamy in one strain of *E. minuta*. From the resulting data, inferences will be drawn relative to the nuclear events occurring during autogamy. As marker genes, those determining the mating types (*mt*) have been used. These are the only

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genes at present available in the strain studied. Previous reports by Nobili (1966*a*, *b*) and unpublished results (Luporini & Nobili, in press) have indicated that at least fourteen different alleles are present at the *mt* locus in this species. At this locus, the alleles can be arranged in ascending order of dominance, from mt^1 (gene determining mating type I) which is recessive to all others, to mt^{14} (gene determining mating type XIV) which is dominant to all others.

2. MATERIAL AND METHODS

The autogamous strain A-25 of the marine species *E. minuta* was studied. This strain was collected three years ago on the Thyrrenian coast near Pisa from eulitoral sand samples and has been maintained since then in flasks under laboratory conditions. In addition, two phenotypically different strains of known genotypes, 5G (mating type XI) and 20 (mating type I), were used for crossing with animals of strain A-25. Neither 5G nor 20 are autogamic strains. The 5G strain is heterozygous at the *mt* locus with an mt^{11}/mt^{10} genotype, while strain 20 is homozygous with an mt^1/mt^1 genotype (Luporini & Nobili, in press*). Under appropriate conditions these three strains could always be induced to conjugate with one another, thus showing that A-25 animals do not belong to either of the other two mating types. Besides the above-mentioned strains, all standard mating types of our collection were also used to test the mating type expressed by the progenies under study.

All strains were cultured on 'Erd-Schreiber-Dunaliella' medium as previously described by Heckmann (1963) for *E. vannus*. Cultures were routinely kept in test tubes at 22–24°C., except the offspring from crosses and autogamy which were permitted to divide at maximum fission rate by reisolating them in depression slides every second day. The cytology of autogamy was studied in animals fixed in either Sanfelice or Schaudinn fixative followed by Feulgen or Giemsa staining. Other technical details will be set forth in the body of the paper.

3. RESULTS

(i) *Cytology*

The cytological analysis of the nuclear events occurring during autogamy was carried out on animals serially fixed from the very onset to the end of the process. To have specimens at various stages, cultures which were yielding 100% autogamous animals were used. It was found that the micronucleus undergoes four successive divisions in the same manner as described above for conjugation. As soon as meiosis is completed, two of the eight nuclei thus formed divide again mitotically, producing the four pronuclei. Two of these four nuclei unite to form the synkaryon. It is impossible to determine by cytological techniques whether these two nuclei are alike or come from different products of meiosis, due to the smallness of the animals (less than 50 μ) and of the nuclei (3 μ in diameter). This problem

* In this paper the new data gathered by analysing eleven non-autogamous mating types are presented to fit the mating-type scheme of the species as reported by Nobili (1966*a*).

can only be solved with the help of the genetic evidence as given below. Hundreds of animals were examined at different stages of macronuclear reorganization (which starts after the synkaryon has mitotically divided twice) up to the first post-autogamous fission. Fusion of fragments of the old macronucleus with the developing new one was never noticed. The above observations are in agreement with the short description of autogamy given by Siegel & Heckmann (1966).

(ii) *Effect of autogamy on segregation of* mt *alleles*

Animals from strain A-25 were passed through autogamy and crossed with strains 5G (mating type XI) and 20 (mating type I). The mating types of exconjugant clones were determined and the results are shown in Table 1. All F1 clones of both crosses, each one derived from a different mating pair, were tested against

Table 1. *Results of crossing and of autogamy with strain A-25*

	Mating types of progeny							
Cross	A-25	XI	X	I	46c	Unclassified	Expected ratio	χ^2
A-25 × 5G*	20	37	15	—	—	—	1 : 2 : 1	0·81
A-25 × 20†	26	—	—	—	27	—	1 : 1	0·04
Autogamy A-25	70	—	—	—	1	1		

* Strain 5G has mating type XI, genotype mt^{11}/mt^{10}.
† Strain 20 has mating type I, genotype mt^{1}/mt^{1}.

the standard types and were assigned to the mating type of the standard, with which they gave no reaction. In the table, the Roman numerals (XI, X, I) refer to the standard non-autogamous mating types to which the examined clones proved to belong. In addition, two new mating types, here referred to as 'A-25' and '46c', were found. From the first cross, A-25 × 5G, a mating-type segregation ratio of 1 : 2 : 1 for types A-25 : XI : X was obtained. From the second cross, A-25 × 20, there was a 1 : 1 segregation ratio for types A-25 : 46c. The observed segregation ratios suggest that true conjugation had taken place between the crossed animals. That nuclear exchange had indeed occurred was confirmed by the fact that all F1 clones, independent of their mating type, had acquired the autogamy trait. It is concluded that the original A-25 animals were heterozygous at the *mt* locus, although they had passed through at least two autogamies. Hence, autogamy does not necessarily result in homozygosity in *Euplotes*.

To test further if autogamy does in fact maintain heterozygosity, fifteen animals of strain A-25 were singly isolated into depression slides and allowed to go into autogamy. From one of the fifteen cultures which first underwent autogamy, eighty autogamous animals were isolated; eight of them died and the surviving seventy-two gave rise to clones which were followed to sexual maturity by transferring them every second day. As the results (Table 1, lower part) indicate, one

clone (13a) out of the seventy-two proved to belong to the same new mating type, '46c', as had been found by crossing stock A-25 with strain 20; of the remainder, seventy expressed the 'A-25' mating type and one could not be classified in respect of mating type before going into autogamy again.

As explained in the section on cytology, three possibilities can be visualized as to which nuclei will fuse together in synkaryon formation. Each possibility has different genetic consequences when heterozygotes are put through autogamy: (1) the two pronuclei which fuse are identical. In this case a 1 : 1 ratio of the mating types appearing after autogamy would be obtained, as in *P. aurelia*. (2) The two nuclei are always genetically dissimilar; then, there would not appear to be any mating-type segregation since all ex-autogamous animals would be heterozygotes. (3) Karyogamy occurs randomly between any two of the four pronuclei formed by any two randomly surviving products of meiosis. In this case a 13 : 16 : 13 segregation ratio would be obtained, e.g., 13 dominant homozygotes, 16 heterozygotes, and 13 recessive homozygotes (Sonneborn & Beheme, personal communication). Because the dominant homozygotes are indistinguishable from the heterozygotes at the *mt* locus, a 29 : 13 phenotypic segregation should be observed. In formulating the expectations on the assumption of complete randomness of nuclei for karyogamy, Sonneborn and Behme considered two steps: (*a*) random survival of any two of the eight nuclei produced at meiosis; (*b*) random survival of any two of the four pronuclei mitotically originated from the two surviving nuclei of step (*a*). There are 28 possible combinations of two nuclei from a set of 8 nuclei. In the case of the 8 products of meiosis from a heterozygote—4 bearing one allele, 4 bearing the other—6 of the 28 combinations would include two nuclei bearing the dominant allele, 6/28 would include two nuclei bearing the recessive allele, and 16/28 would include one nucleus with the dominant and one nucleus with the recessive allele. When both surviving nuclei bear the dominant, all the pronuclei must also bear the dominant allele and the synkarya will be homozygous dominant. This accounts for 6/28 of the autogamous cells. In like manner, 6/28 would be homozygous recessive. These two classes will be augmented by some coming from the remaining class, i.e. the 16/28 in which one dominant and one recessive nucleus survive after meiosis. The random selection of the two functional gamete nuclei among the two dominant and two recessive nuclei present after the surviving nuclei go through one mitotic division yields both dominant to both recessive to one of each in the ratio 1 : 1 : 4. Hence, 1/6 of 16/28 or 2/21 of the autogamous animals become homozygous dominant and the same proportion becomes homozygous recessive by this route, the remaining 8/21 becoming heterozygous. The total proportion of homozygous recessives is thus $2/21 + 6/28$ or 13/42, the remainder (29/42) being dominant, 13/42 homozygous and 16/42 heterozygous.

Since only one clone out of the seventy-one instead of the twenty-two expected (Table 1, lower half) could actually be presumed to be the recessive homozygote, these results rule out the first and the third possibilities and suggest that the second hypothesis is the most probable one. The seventy ex-autogamous clones expressing the A-25 mating type could either all have become homozygous for the dominant

mt gene or else become a mixture of homozygotes and heterozygotes in the ratio of 13 : 16 after autogamy. This was not the case, as shown by the next experiment.

The clone expressing the new mating type '46c' and twelve randomly chosen clones of the seventy with 'A-25' mating type were crossed with animals of strain 20 (which is homozygous mt^1/mt^1, the most recessive of the alleles). Some pairs from each cross were isolated and one clone of each pair was followed to sexual maturity. All clones were tested against the standard mating types I, 'A-25', and '46c'. The results are presented in Table 2. As expected, all eighty-two clones obtained

Table 2. *Results of crossing ex-autogamous clones from strain A-25 with strain 20*

	Analysis of progeny clones			
Mating type of strain A-25 parent	No. tested	Mating types I	A-25	46c
46c	82	—	—	82
A-25	38	—	23	15
,,	37	—	18	19
,,	33	—	12	21
,,	30	—	15	15
,,	31	—	14	17
,,	31	—	15	16
,,	38	—	23	15
,,	41	1	21	19
,,	37	—	21	16
,,	40	—	26	14
,,	32	2	11	19
,,	41	—	18	23

by crossing clone of type 46c with strain 20 showed again the mating type '46c', thus confirming that this clone was homozygous at the *mt* locus. The progenies from the twelve crosses between ex-autogamous A-25 clones and strain 20 showed an approximate 1 : 1 ratio of types 'A-25' and '46c'. Three clones however expressed mating type I (Table 2, second column). These can be explained as due to cytogamy in the strain 20 conjugants. (Cytogamy is a process of internal self-fertilization in animals which appear to conjugate, but which in fact do not exchange nuclei (Wichterman, 1940).) In any case the data in Table 2 show that all twelve of the A-25 clones tested were heterozygous at the *mt* locus, instead of sixteen out of twenty-nine expected on a basis of random fusion of gamete nuclei (see above). Although the numbers are small, these results favour the view that autogamy here results preferentially in the formation of heterozygotes.

Further evidence in favour of this hypothesis was obtained from a study of the autogamous progeny of three different hybrid clones (A-25 × 5G; A-25 × 20; and 13a × 20). The heterozygous state of the three F1 clones was ascertained by crossing them with strain 20. A low survival value ranging from about 4 to 19 %, was found

among the many hundreds of animals isolated after autogamy from these three clones. Such a high mortality rate is very common only in the F2 from 'hybrids' between autogamous and non-autogamous strains (Nobili, 1966*a*; Siegel & Heckmann, 1966). As the results show (Table 3), only three out of a total of 104 ex-autogamous clones examined yielded descendants of the expected recessive types.

Table 3. *F2 clones obtained after autogamy of three genetically dissimilar F1 clones*

Genotypes of F1 clones	Mating type of F1 clones	No. of ex-autogamous clones examined	Mating-type segregation after autogamy			
			A-25	X	46c	I
mt^{10}/mt^{46c}	X	28	—	27	1	—
mt^{A-25}/mt^{1}	A-25	39	39	—	—	—
mt^{46c}/mt^{1}	46c	38	—	—	36	2

If we assume an equal survival probability of the mating types which should arise at the autogamy, these results are explicable on the basis of preferential karyogamy. However, the heterozygous state of these F2 which had passed through autogamy without changing mating type could not be checked. Therefore, macronuclear regeneration cannot be excluded and may even account for the persistence of heterozygosity in the few survivors after autogamy. (Macronuclear regeneration is the formation of the new macronucleus from fragments of the old macronucleus.)

The findings reported here all point to a preferential fusion between genetically different nuclei during autogamy resulting in a preponderance of heterozygotes.

(iii) *Effect of conjugation on segregation of* mt *alleles*

Does this preferential karyogamy also occur during conjugation involving A-25 animals? Such a question can be easily answered by analysing the inheritance of mating type in the descendants of both conjugants of each pair. If karyogamy occurred at random during conjugation, we would obtain an 8 : 13 segregation ratio of clonal and synclonal mating-type inheritance, as we have seen in the preceding section unless cytogamy occurred. (By 'synclonal inheritance' we mean here that the two ex-conjugant clones from a given pair of conjugants are genetically identical; if there is 'clonal inheritance' the two ex-conjugant clones may differ.)

An A-25 mating-type clone of the seventy which had passed through autogamy (Table 1, last row) was crossed with strain 20. Sixty pairs were isolated and forty-nine synclones were examined as to their mating type. The results are shown in Table 4. Because cytogamy was not found in the forty-nine pairs examined, the deviation from the expected segregation ratio of 13 : 8 of clonal and synclonal mating-type inheritance is significant at the 5% level, not at the 1% level on the assumption of random karyogamy.

Table 4. *Numbers of pairs from cross of A-25 animals with strain 20 animals, showing clonal and synclonal mating-type inheritance*

	Pairs with synclonal inheritance	Pairs with clonal inheritance	χ^2
A-25 mating type	15		
46c mating type	8		
	23	26	4·64

'Synclonal' inheritance is found when both ex-conjugant clones from a pair show the same mating type; 'clonal' inheritance is found when the two ex-conjugants differ.

Thus, the direction of deviation points to preferential karyogamy between dissimilar nuclei even during conjugation of A-25 animals, but more data are needed for actual confirmation. Still, these results show that synkaryon formation appears to be different at autogamy and conjugation in strain A-25.

4. DISCUSSION

The results presented indicate that during autogamy karyogamy between genetically dissimilar nuclei occurs more frequently than expected on a random basis; the genetic consequence is to maintain heterozygosity in those animals which undergo autogamy. Fertilization nuclei derived from different products of meiosis in the two ex-conjugants have been known to occur in conjugating euplotes of certain species. Heckmann (1963) was the first to analyse this phenomenon in *E. vannus*, in which he found it happening in 174 out of 530 mating pairs examined. In *E. crassus* (Heckmann, 1964), its occurrence, analysed in only one cross, was found in 37 out of 103 pairs examined. In *E. minuta* (Nobili, 1966*a* and unpublished results), 10% of the 735 pairs examined showed their synkarya to have originated from genetically dissimilar nuclei. While the results concerning the first two species are roughly those expected on the assumption of complete randomness of gamete nuclei taking part in synkaryon formation, the data from *E. minuta* indicate that synkaryon formation is brought about, more frequently than expected on a random basis, by karyogamy of genetically identical pronuclei. These data are derived from crosses in which no autogamous strain was involved. In fact, in conjugating A-25 animals of *E. minuta*, any two of the four pronuclei may function at random in synkaryon formation. Such behaviour seems at variance with what observed in conjugating non-autogamous animals. However, it may well be that this peculiar behaviour of nuclei during both conjugation and autogamy in stock A-25 does not occur in other autogamous strains of the species. This needs to be investigated more fully before stating any general conclusion relative to differences between autogamous and non-autogamous strains bearing on synkaryon formation.

Autogamy is the most intense form of inbreeding known to occur in some ciliates. According to Sonneborn (1957), autogamy, in *P. aurelia*, results in conditions approaching those associated with haploidy, since there is no hidden recessive masked by dominant alleles, all loci being homozygous. Genetic variability is

lacking after autogamic reproduction, although it can be immediately restored through conjugation due to the fact that no immaturity period follows the sexual process of autogamy in this species. In *E. minuta*, at least in strain A-25, autogamy seems to be at least as effective as selfing in maintaining heterozygosity, the basic requirement for bringing about genetic recombination. Moreover, if we consider the fact that autogamy in this strain is able to maintain a heterozygous state originating from crosses of autogamous with non-autogamous strains, we can readily recognize its effectiveness in producing genetic variability in spite of the *per se* inbreeding characteristics. These facts distinguish the autogamic process of *E. minuta* from that occurring in *P. aurelia*. Autogamy in the two species appears to be as different as parthenogenesis, particularly the haploid one, from hermaphroditism in self-fertilizing metazoan organisms as far as the genetic consequences of the two processes are considered. Of course much more needs to be known about the breeding system prevailing in nature in *E. minuta* and the extent of the apparent divergence between the autogamous and non-autogamous strains as indicated by their high death rates at the F2, before drawing any conclusion as to the evolutionary significance of autogamy in this species.

SUMMARY

The genetic consequences of autogamy have been analysed in the autogamous strain A-25 of *E. minuta*. During autogamy as well as during conjugation four pronuclei (two genetically identical pairs) are formed in each individual. In conjugating pairs, any two of the four pronuclei of the autogamous conjugant may participate in synkaryon formation. Consequently, an 8 : 13 segregation ratio of clonal and synclonal mating-type inheritance has been found to occur in the progeny obtained by crossing A-25 animals with non-autogamous animals. During autogamy, synkaryon formation appears to be brought about by a preferential karyogamy of genetically dissimilar nuclei more often than is expected on a random basis. Therefore, heterozygosity is usually maintained after autogamy in spite of the extreme inbreeding characteristics of this sexual process.

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