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Preliminary results of genetic analysis in *Ascobolus immersus*

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G. Rizet gave in 1939 the first indication that *Ascobolus immersus* is a suitable object for genetic studies. In recent years Rizet and his collaborators published some of the results of their work on the genetics of *A. immersus*. (Rizet 1958, et al. 1960, Lissouba et Rizet 1960, Chevaugeon 1959 a, b, c).

A. immersus is a coprophilous fungus of the class *Ascomycetes (Discomycetes, Pezizales, Ascobolaceae)*. The main advantage of this heterothallic fungus is that the ascospores are relatively large and are ejected from the asci in groups of eight. Spontaneous mutations are frequent and affect, among other, the morphology of the ascospores; this enables direct analysis of tetrads. *A. immersus* grows quite easy in laboratory conditions, its developmental cycle is short. The present communication is a preliminary report on the results of experiments so far carried out.

MATERIAL AND METHODS

Experiments have been carried out on strains of *A. immersus* received from Rizet and with his advice and help. I would like to take this opportunity to express my gratitude to prof. Rizet for his kindness*.

In the laboratory the developmental cycle of *A. immersus* lasts about 12 days. The mycelium grew well in an artificial medium according to Yu (1954):

Yeast extract — 0,4%
Agar — 2,5% pH about 6,3

* Professor Georges Rizet generously permitted our laboratory to benefit from his great experience in this field. Not only did he send us a great deal of his experimental material, but he also helped us in many other ways too: he mailed us many technical instructions of the special methods of culture he employs, and also allowed one of our staff members to study with him for three months. For all the assistance and help he has given us mere formal thanks are too small.

Crosses were made on sterilised horse dung. Beginning from 4 days after crossing the dishes were exposed to light for at least eight hours a day. A temperature of 26°C was maintained, and the air was kept moist. The apothecia emerged after 5—6 days, the ascospores began to ripe after 9—11 days. Beginning with the 9th day the dishes are covered with agar slides (agar — 2,5%) and the slides are changed daily until the 20th or 21st day after crossing. The groups of eight ascospores which are ejected and stuck to the agar slides were analysed under a dissecting microscope. From a single dish, 6 cm. in diameter anything from a few ascospores to a few thousand groups of eight ascospores were found. Rizet's method of germinating the ascospores was used (peptone — 1,25%, agar — 1,25%) but we also alkalised the medium by adding 0,15% of NaOH. On this medium a temperature shock of 39—40°C over a period of 16 hours was applied. In 1923 Schweitzer who used a temperature shock (5—6 hours, 38—40°C) working with *Ascobolus citrinus* growing on rabbit dung explained that this is an adaptation to body temperature and the rabbit's period of digestion. After germination the mycelia were moved to the artificial medium and the next day it was possible to use them for further crossing.

The material used was one wild form and 11 mutants.

A mature ascus of the wild form contains eight violet-brown spores of 50—70 × 30—35 μ. They are stuck together because of the jelly-like substance surrounding them. The whole group of eight ascospores is suspended beneath the upper part of the ascus (Zopf 1880) and at a certain moment is shooted up like a bullet. According to Buller (1909) the force of the shot may carry it to 35 cm.

The mutants used differed from the wild form in the morphology of their ascospores in the following ways:

1) one granular mutant — the pigment of the spores is not smoothly spread, but forms smaller or larger granules (mutant XX);

2) one rough mutant — the membranes of the ascospores are rough (mutant VII);

3) nine white mutants — the ascospores are unpigmented, white (mutants: E, 70, 78, 113, 184, 185, 164, 186 and 131). The numbers of mutants are from Rizet's laboratory.

RESULTS

The results of 74 crosses and the analysis of more than 60 thousand groups of eight ascospores are presented here. The crosses which did not produce at least 300 analysable asci were usually not taken into account.

All the mutants were monogenic in accordance with the findings of Rizet (1960); in crosses with the wild form they gave a segregation of

4 mutants and 4 wild types. The differences between the white mutants were also in agreement with Rizet's findings; they belong to different complex loci, "series", as he calls them. It is quite probable that each different series causes a different blocking of the complex pigment formation process (M. Le Gall 1942). With exception of the crosses: rough × granular and rough × white the results of which were not conclusive it is possible to establish the basic types of segregation in all other crosses. However, in all combinations irregular segregations also appeared.

Regular segregation

a. White mutants × white mutants

Rizet classified white mutants we worked with in the following way:

E	— belongs to one series,
70, 78, 113, 184, 185	— belong to a second series,
164	— „ „ third series,
186	— „ „ fourth, linked with 164,
131	— unclassified.

Crosses: 78 × 113 and 113 × 70 reveal that these three mutants really belong to the same series; not counting rare exceptions, about which we shall speak later, all the asci gave eight white ascospores (symbol — 8:0).

In crosses between mutants from different series many wild recombinants appeared. This means that these mutants are not allelic, that again they do belong to different series. The same applies to the mutant 131: it belongs to some other series.

As far as the relationship between different series is concerned, if they are linked or independent one can assess this by counting the number of different types of tetrads. Table 1 gives the results of nine crosses between mutants belonging to four different series.

There are three types of asci:

8 : 0, parental ditypes (PD) containing two kinds of spores, both parental,

6 : 2, tetratypes (T) with four kinds of spores, two parental and two recombinant,

4 : 4, non parental ditypes (NPD) with two kinds of spores, both recombinant.

To test the independence of the four series from Table 1 Catcheside's (1949) provisory method was employed, two criteria were simultaneously verified: the relationship between PD and NPD and the recombination percent. The first should not deviate from 1 : 1, the second —

from 50%. As one sees in Table 1 all the crosses show independence. The t test was used instead of χ^2 which is usually used.

Table 1 shows deficiency of PD (8 : 0) in crosses: 1, 2, 6, 7, 8 and 9. This deficiency is particularly noticeable because groups of eight white spores appear often, as we shall see later, as a phenotypical modification and an excess of them should be rather expected.

Table 1

Crosses between mutants of different series

Entry no.	Cross	Regular segregation				PD : NPD 1:1		% recomb.
		PD	NPD	T	Total Groups of eight	t	P	
1	186×113	196	258	699	1153	0.8	0.4	52.7
2	E× 70	518	746	1922	3186	2.0	0.05	53.4
3	113×E	527	443	1640	2610	0.9	0.4	48.4
4	186×185	106	83	338	527	0.6	0.5	47.8
5	185×131	69	66	205	340	0.1	0.5	50.0
6	186×131	278	367	1324	1969	1.3	0.2	52.3
7	E×131	564	668	2691	3923	0.9	0.4	51.3
8	E×185	54	66	353	473	0.4	0.5	51.0
9	184×186	420	538	1889	3157	1.2	0.2	48.5
	Total	2432	3235	11061	16338			

To find out, whether or not, this is caused by the fact that in different types of asci the ascospores stick together more or less firmly, in one of the crosses not only the groups of eight, but all the spores were counted. Table 2 shows the results of this counting in 10 samples from cross 184 × 186. When counting only the ascospores deriving from incomplete groups deviations are rarer and smaller than when counting only the complete groups of eight spores; when adding both numbers together the deviations are smaller. However these results do not prove that the ascospores from incomplete groups account for the deficiency of PD in the groups of eight. Perhaps this difference is also accounted for by the fact that certain types of ascospores are ejected more easily depending whether or not they are all mutants, or at least some of them are wild. Anyhow, these results show the necessity of supplementing the results of groups of eight (i.e. tetrad analysis) with the results of all the spores, i.e. random sample method. This will be of the utmost importance during the later stages of work on *A. immersus* when a greater number of known loci and linked groups will enable one to work out genetic maps using more precise

methods of estimating (Papazian 1952, Perkins 1949, 1953, 1955, Barratt et al. 1959, Shult and Lindegren 1956).

In crosses between mutants of different series one observes that the frequency of tetratypes changes during the experiment. All the groups of spores were divided into three successive parts and the percentage of tetratypes was counted in each part.

Table 2

Comparison between tetrad analysis and random sample method

Number of sample from cross 184×186	Complete groups of eight spores				Spores from incomplete groups			Sum of all spores Deviation from 3:1 χ^2
	PD	NPD	T	Deviation from 1:1 χ^2	white	wild	deviation from 3:1 χ^2	
1	7	7	22	0.0	84	41	4.0	1.2
2	11	21	62	3.1	308	118	1.6	4.5
3	50	103	294	18.3	492	159	0.1	13.2
4	46	45	173	0.01	673	230	0.1	0.0
5	53	76	245	4.1	230	76	0.0	3.3
6	29	37	164	1.0	279	67	5.9	0.0
7	45	63	227	3.0	342	123	0.5	3.1
8	49	100	334	17.4	381	94	6.9	7.3
9	77	37	202	14.5	193	62	0.6	12.8
10	52	46	155	0.4	175	44	2.8	4.4
	419	535	1878	14.0	3157	1014	1.6	8.5

Table 3 shows the results of counts made in this way on seven crosses. In all these crosses the percentage of tetratypes increased when comparing the first part with the third, and in six out of the seven an increase was noted when comparing the first part with the average. While statistically the difference was not significant, it was shown, however, that the trend is towards an increase in tetratypes during the period of fructification. As the period of fructification continues the conditions in the medium change, the meiotic divisions which precede the formation of first ascospores happen in different conditions from those in which the last spores are produced. This may perhaps cause the difference in the frequency of crossing-over and therefore in the frequency of tetratypes. Similar findings have been reported many times (e.g. Levine 1956). May be *A. immersus* would prove to be suitable for such kind of studies.

As previously stated mutants 164 and 186 are linked. However, I did not succeed in getting many asci from direct crosses, but a cross: 186 × (164—185) i.e. with a double mutant derived from 164 × 185, produced the following types of asci: PD — 168, NPD — 10, T — 112.

Table 3

Percentage of tetratypes

Entry	Cross	No. of groups of eight spores	Tetratype %			
			first part	second part	third part	average
1	186×113	1158	54.6	63.4	63.6	60.4
2	E× 70	3206	55.4	60.0	65.2	60.0
3	113×E	2630	60.6	62.2	63.2	62.4
4	186×185	527	65.0	61.8	65.6	64.2
5	185×131	341	56.2	56.6	65.6	60.0
6	186×131	1978	65.2	66.8	68.6	67.0
7	E×131	3960	67.6	64.0	73.2	67.8

The great excess of PD over NPD shows linkage; in case of linkage NPD can appear only after four strands double crossing-over.

So far we have dealt only with crosses of white mutants within one series or between different series. We shall now turn to the crosses within the same mutant (between + and —). In such crosses two mutants, namely E and 186 produce a great number of groups of eight of the type 8 : 0, with only white spores. Mutant 164 is quite fertile but gives a very high percentage, up to 13, of asci with wild and granular ascospores. In spite of many attemps I did not succeed in getting apothecia from mutants 113, 184 and 185. The same applies probably to other mutants in this series and to the mutant 131 with which not so many tests were done. These results require further studies for it is not easy to state if the sterility of *A. immersus* is genetic or merely caused by external conditions.

b. White mutants × Granular mutants

A granular mutant was crossed with two white mutants and with one double white mutant. A cross was also made between a double mutant, white and granular, with a wild form. Apart from the white and granular ascospores these crosses produced wild spores, even when neither of the parent was of the wild type. No spores which were both granular and white were found. This seems to indicate that when there is a lack of pigment, one cannot distinguish the pigment granules. The granular mutant may be caused by the fact that the pigment is not evenly spread. Further tests also led to the same conclusion: crosses between granular and white produced double mutants which were both white and granular having a white phenotype.

Comparison between crosses 185 × XX and 164 × XX (Table 4, comb. 1st and 2nd) shows that mutants 185 and XX are rather independent whereas 164 and XX seem to be linked. The last assumption is confirmed by the results of cross wild × (164—XX) (Table 4, 3rd comb.) where the excess of PD over NPD is quite clear and the recombination percent is near to that observed in cross 164 × XX.

In the three combinations mentioned above tetratypes 4 : 2 : 2 (4 white : 2 wild : 2 granular) were found instead of tetratypes 2 : 2 : 2 : 2, because the granular spores when unpigmented are impossible to distinguish from the white ones. In the 4th combination, where a double white mutant is crossed with a granular one, apart from tetratypes 4 : 2 : 2 following tetratypes also appeared:

6 white : 2 granular — 152 asci.
6 white : 2 wild — 116 asci.

Considering the difference between numbers of these two types of asci one may estimate the recombination percent between 164 and XX; if mutants 164 and XX were not linked the numbers should be equal, the existing difference indicates linkage. Assuming the number of 152 asci corresponds to free recombination (50%) then the number of 116 asci corresponds to 38,2% of recombination, which quite agrees with the results of other crosses from Table 4.

Table 4

Crosses between white and granular mutants

In tables showing segregation types the first number denotes white spores, the second — wild ones
To indicate granular spores the letter „g" is added

Entry no.	Combination	No. of crosses	Regular segregation types							Percent recomb
			4:4g	4:2:2g	4:4	6:2	6:2g	8:0	Total groups of eight	
1	185×XX	1	PD 43	T 119	NPD 49	—	—	—	211	51,4
2	164×XX	4	PD 331	T 528	NPD 89	—	—	—	948	37,2
3	wild ×(164–XX)*	6	NPD 297	T 2371	PD 1277	—	—	—	3945	37,5
4	XX×(185–164)**	2	PD 246	T 371	NPD 44	T 116	T 152	T+NPD 360	1282	38,2

Footnotes:
* Indicates a double mutant deriving from cross 164 × XX.
** Indicates a double mutant deriving from cross 185 × 164

Irregular segregation

By irregular segregation it is referred to exceptional tetrads and not to deviations from the expected numbers of different types of asci. In Table 5 one sees that asci irregular as far as pigment content is concerned, appear in all possible groupings of eight spores, namely: 8 : 0, 0 : 8, 7 : 1, 1 : 7, 6 : 2, 2 : 6, 5 : 3, 3 : 5 and 4 : 4. Besides these, asci with granular spores appeared. The different irregularities do not appear with the same frequencies; e.g. asci of the type 8 : 0 are frequent, whilst asci 3 : 5 are very rare. The frequency of irregularities varies in different kinds of crosses; they are often in the cross 164 × 164 and very rare in other crosses of the same combination. An attempt was made to assess the various factors which might cause these phenomena. These may be either mistakes in classification or new mutations. After eliminating these causes a category which needs explanation may be found.

Table 5

Crosses between wild strains

Cross	Regular segreg. 0:8	Irregular segregation						No. of groups of eight	Mut. %
		8:0	5:3	6:2	2:6	with gran. spores	4:4		
wild × wild	443	9				1	1	454	0.2
wild b × wild o	927	28				2	3	960	0.3
wild b × wild i	1592	11	1				16	1620	1.0
wild b × wild p	1390	10					3	1403	0.2
wild b × wild w	332	1		3	2		3	341	1.0
wild b × wild s	705	14		1	1		3	724	0.5
wild 2 × wild i	216	5		1			2	224	1.0
wild b × wild y	127	4						131	0.0
wild b × wild n	1709	12				6		1727	0.0
wild e × wild i	371	12					1	384	0.2
wild m × wild i	271	9						280	0.0
Total	8083	105	1	5	3	9	32	8238	

Misclassifications

Sometimes it is not easy to distinguish a wild but weakly pigmented ascospore from a white one. Occasionally one observes in wild type ascospores a whole gamut of colours, from normal violet-brown to pale pink, these differences occuring both within groups and between them. It seems that the reduced intensity of pigment is caused by an increase in moisture of the medium, however Rizet (1960) states that this may be because

there are also weakly pigmented mutants. However, such difficulties in scoring white and wild ascospores which could lead to misclassifications for instance of the type 6 : 2 instead of 4 : 4 were rare and even then appeared only in certain crosses.

Another cause of errors in classification is the possibility of ascospores from different asci getting joined together. Ascospores not always are ejected together, in groups of eight, sometimes they are in smaller groups or single. This may cause spurious octads which really derive from more than one ascus. The probability of such an event seems to be rather slight, it can, however, explain certain rare exceptions.

Another cause of error is the overlapping of generations. During the early stages of our work on *A. immersus*, when we analysed the dishes up to the end of the fructification, instead as we do now, up to the end of the twenty first day, we often found irregularities. These were: asci with eight granular ascospores, when only one of the parents was granular or eight wild when the expected segregation was 4 : 4. These irregularities were simply caused by the overlapping of generations. Winge (1954) made similar findings in yeasts. Perhaps this explain the rare cases in which asci of the type 0 : 8 were found.

New mutations

In crosses within wild strains new white mutants appear. They are found in asci of the type 4 : 4. In Table 5 where all kinds of irregular segregation found in 11 crosses of different wild strains are shown, the frequency of such mutants may be observed. The percentage of asci in which new mutants appear varies from 0 to 1. This is in agreement with Rizet's (1960) opinion concerning the high rate of spontaneous mutations in *A. immersus*: at the same time the results show that differences may exist between various strains. The granular spores which appeared in these crosses were not tested; it may well be, that they are too new mutations.

Other irregular segregation

As one sees from the above, errors in classification and new mutations could account for all observed types of irregular segregation. However, the frequency of irregularities is sometimes so high that further explanation seems necessary. In Table 6 where the appearance of irregularities in all crosses is presented, one can see the high frequency of type 8 : 0 in almost all combinations. It must be added that in all these combinations where type 8 : 0 is expected, all the asci of that type were classified as

regular. Ascospores from such asci gave often rise to wild strains, which shows, that they present phenotypical modifications. The pigment in the spores appears fairly late on; it is not until just before they are ejected, that one sees pigmented spores in the asci — up to that time they are quite white. Perhaps it is an excess of moisture in the medium which causes them to spring up prematurely; for when there is an excess of moisture the type 8 : 0 seems to occur more frequently.

Another striking point in the Table 6 is the behaviour of mutant 164 when crossed + with — (combination 3). In such a cross the percentage of irregular asci goes up to 13. The most frequent classes of the irregular asci were of the types 7 : 1 and 6 : 2 where the exceptional spores were of the wild type or granular. In the cases when they were tested, they behave according to their phenotypes. It is as yet difficult to account for their origin.

The most remarkable case in Table 6 concerns the crosses: wild × 186 and wild × E, which produce irregulary asci of the types 6 : 2 and 2 : 6 with a frequency of 4 to 6%. Other white mutants when crossed with wild type show a very low frequency of such asci. So far only few of these exceptional asci were genetically tested: a part of them shows segregation 3 : 1 (in asci of the type 6 : 2) or 1 : 3 (in asci of the type 2 : 6), in other normal segregation 2 : 2 was found.

As yet the identity of either six white spores or six wild ones is not proved. It is interesting that the mutants E and 186 are the same which are fertile when crossed within mutants on the contrary to other white mutants we worked with.

DISCUSSION

The two exceptional types, 6 : 2 and 2 : 6, were studied with Rizet's recent findings in mind (Lissouba et Rizet 1960, Rizet et al. 1960, unpublished), These should be presented briefly. When crossing white mutants belonging to the same series, few wild ascospores appear, with the frequency up to 1% in type 6 : 2 asci. In several series the mutants were arranged according to their recombination frequency.

From one of these series 120 asci of type 6 : 2 were genetically tested. The tests showed that it was not conventional crossing-over in either of these asci; no double mutants were found and an uneven number of parental spores appeared in each ascus: 4 were of one parental type ("majoritaire", segregation 2 : 2) and 2 of the second parental type ("minoritaire", segregation 1 : 3). Basing on these facts Rizet assumed that he was dealing with a recombination of conversion type without

Table 6

Irregular segregation

Entry no.	Crosses		No. of crosses	Groups of eight spores total	Regular segreg.	Irregular segregation										
						8:0	0:8	7:1	1:7	6:2	2:6	5:3	3:5	4:4	With gran. spores	Irregul. segreg. total
1	wild × wild	between diff. strains	11	8238	8083	105				5	3	1		32	9	155
2	wild × white	between wild and 70, 78, 113, 185, 164	9	5743	5602	107		1		8	4	6	2		13	141
		wild × 186, wild × E	8	8590	7687	134	8		1	362	324		2		72	903
3	white × white within mutant	186 × 186, E × E	5	3135	3135					1						1
		164 × 164	2	1660	1449			117		65		14		7	8	211
4	white × white within series	78 × 113	1	858	857		•	1								1
		113 × 70	1	1295	1302			2		3					2	7
5	white × white between series	186 × 113, E × 70, 113 × E, 186 × 185, 185 × 131, 186 × 131, E × 131, E × 185, 184 × 186 164 × 185.	13	17345	17258			3	1	3	2	1	1		76	87
6	wild × (white-white)	wild × (164 — 185)	2	384	383										1	1
7	white × (white-white) two mutants	(185 — 186) × 185	1	677	668			8		1						9
		164 × (185 — 164)	1	702	712			16		3					1	20
		(185 — 186) × 186	3	1220	1217					3						3
8	white × (white-white) three mutants	186 × (164 — 185)	1	290	290											—
9	granular × granul.	XX × XX	1	1118	1100	18										18
10	wild × granul.	wild × XX	2	2103	1984	47	42		27			29		19		119
11	white × granul.	185 × XX, 164 × XX	5	1221	1159	43				4 11g		2 1g	1			62
12	granular × (white-white)	XX × (185 — 164)	2	1289	1282		1				2	2	2			7
13	wild × (white-granular)	wild × (164 — XX)	6	4075	3950	117	1			4						125
		Total	74	60011	58142											1870

reciprocal exchange. One may use the following scheme of asci to explain this:

+ a	+ a	
+ a	++	
b +	b +	where b segregates 2 : 2
b +	b +	a segregates 1 : 3
tetrad	tetrad	
without conversion	with conversion	

For each pair of crossed mutants it is always the same mutant which shows segregation 1 : 3 but the same mutant which is "minoritaire" in one cross may be "majoritaire" when crossed with another mutant.

The pattern of segregation can be predicted when knowing the arrangement of mutants, for in every pair the segregation 1 : 3 happens always on the same side. The results agree both when taking into account the recombination frequency and when considering the "minoritaire — majoritaire" relationship.

Apart from the series discussed above Rizet studied other series which revealed two different kinds of asci of the type 6 : 2; one as above and a second which was the product of conventional crossing-over. This shows that the other series are more complex. Basing on these facts Rizet proposed a new genetic unit connected with the peculiar recombination type; this unit he called "polaron" on account of its polarity. Polaron is a polarised linear unit within which recombination is only of the conversion type. Rizet is seeking reverse conversion too, i.e. cases in which segregation would be 3 : 1 more or less according to the following scheme:

+ a	+ a	
+ a	b a	
b +	b +	where a segregates 2 : 2
b +	b +	b segregates 3 : 1
tetrad	tetrad	
without conversion	with conversion	

After such conversion an ascus containing among other ascospores a pair of double mutants would consist of all white spores and it would be therefore impossible to distinguish it without genetic tests. Such asci are not found as yet. To overcome this difficulty Rizet attempted to cross different white mutants with the wild form, looking for asci of the types 6 : 2 and 2 : 6 — and in fact they did appear.

Types 6 : 2 and 2 : 6 which appeared in our experiments were mostly from crosses between wild type and two white mutants: E and 186. Only after additional tests it will be possible to conclude that we are

dealing with a truly genetic change. If this will be proved to be a fact then we could assume a conversion-type mechanism; copy choice without reciprocal exchange. Crossing over can be discarded because after conventional crossing-over we should expect normal 4 : 4 segregation. It is also difficult to conceive of an arrangement of supressors producing such a high and even frequency of types 6 : 2 and 2 : 6.

It is as yet too early for any generalisations and further studies are necessary. It is important to find out what are the differencies between series and between mutants from the same series when crossed with wild type. It is also essential to ascertain, testing the exceptional asci, whether or not the products of the miscopying are really copies of homologous sites i.e. to find out if the six spores, wild or white, are identical. At the same time it is necessary to learn about the life cycle of *A. immersus*, and among other to distinguish between genetic sterility and lack of apothecia caused by external conditions. No less important is the knowledge about the processes prior to the development of asci (Mitchell 1960 a, b).

SUMMARY

74 crosses between 12 strains of *Ascobolus immersus* were performed. The strains we worked with were 1 wild form, 9 white mutants with unpigmented ascospores, 1 granular mutant with granules of pigment on the ascospores and one rough mutant having a rough surface of ascospores. More than 60 000 groups of eight spores were analysed. In the majority of cases regular segregation was stated agreeing and extending Rizet's findings concerning the monogenity of mutants, their division into series and linkage relations. In crosses between white mutants of different series a deficiency of asci of the type 8 : 0 and an increase of the tetratype frequency in time of fructification were observed. In many crosses numerous irregular tetrads are found. After discussing presumable sources of errors, special attention was paid to exceptional asci of the types 6 : 2 and 2 : 6 which arose with a high and even frequency in crosses between two white mutants and a wild form. The possibility to explain these types of asci by conversion is discussed.

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