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Sexual and parasexual analysis of *Ustilago violacea*

By A. W. DAY* and J. K. JONES
Department of Agricultural Botany, University of Reading

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1. INTRODUCTION

The anther smut fungus, *Ustilago violacea*, is eminently suitable for genetical studies. An account of the life-cycle and of the production of vegetative diploids in culture has been published (Day & Jones, 1968), and the paper also outlined methods for the production of diploid and haploid segregants. Diploid strains differ from haploid strains both in the appearance of the colonies and in the size and shape of the cells (sporidia). They are neutral in mating type, solopathogenic, i.e. pathogenic as a pure culture, and genetically stable, spontaneous segregation being so rare as to be negligible. Haploidization can be induced with DL-*p*-fluorophenylalanine (PFP) and mitotic crossing-over induced by irradiation with ultraviolet light (u.v.).

This paper reports the analysis of the genotype of the species primarily from a study of induced haploidization but also from data obtained after meiotic segregation and from complementation tests. The process of haploidization in *U. violacea* will be described and analysed in more detail in a later paper.

2. METHODS AND MATERIALS

The details of the life-cycle, stocks, mutants, media and general techniques have been published previously (Day & Jones, 1968). The techniques for genetical analysis are described more fully below.

(i) *Complementation tests*

The formation of diploid colonies from two compatible auxotrophic haploid mutants, by the method described previously (Day & Jones, 1968) was used as evidence that the mutants complemented and were therefore probably in distinct genes. Failure to produce diploid colonies was regarded as evidence that the mutants were allelic.

(ii) *Haploidization*

A stock solution was made up containing 0·3 g DL-*p*-fluorophenylalanine (Koch–Light Laboratories) in 10 ml distilled water. A pellet of sodium hydroxide was added to form the soluble sodium salt. This solution was sterilized in a 50 ml beaker by a 15 min exposure at a distance of 12 cm from a Camag TL900 u.v.

* Present address: Department of Botany, University of Western Ontario, London, Ontario.

lamp fitted with an 8 W Sylvania tube (G 8T 5) with a wavelength peak of 254 mμ. The 10 ml sterilized PFP solution was added to 1 l. of molten autoclaved complete medium, and a thick layer of PFP medium was poured into each Petri dish as it was necessary to incubate these plates for 3–4 weeks.

About 10^5 cells from a pure diploid colony were streaked all over the surface of the PFP medium, and the plates were incubated for 3–4 weeks. At the end of this time the majority of the cells had stopped growing, but isolated cells had formed scattered spherical colonies (papillae) above the background of dead cells. These papillae were of at least three different types, but over 70 % were of the type illustrated previously (plate 1, fig. 6, Day & Jones, 1968) and were considered to be haploid as they had haploid-sized cells and a positive mating-type reaction with one of the stock haploid lines. The genotype provided further evidence that these papillae were haploid, or near haploid. The other types of papillae and the phenomena associated with the production of papillae will be described in a later paper.

After 3–4 weeks incubation on PFP medium, papillae were picked off carefully from the background of diploid cells and were placed in rows on a fresh plate of complete medium. After incubation for 2–3 days these plates (master plates) were replicated to a series of test media. The test media used depended on the genotype of the diploid under investigation, but each series comprised: (i) a plate of minimal medium; (ii) a plate of minimal medium to which all the supplements had been added; and (iii) a series of plates of supplemented minimal medium from which each one of the supplements was omitted in turn.

(iii) *Meiotic segregation*

Crosses between two haploid stock cultures were obtained by sampling the diploid brandspores produced in the host plant, *Silene alba*. Seed obtained from several botanic gardens was all found to be hybrid (*S. alba* × *S. dioica*). As *S. dioica* is resistant to the strain of *U. violacea* obtained from *S. alba* (Goldschmidt, 1928), many of the inoculations made using plants obtained from this hybrid seed were unsuccessful. Seed obtained from areas in Berkshire, England, where *S. dioica* is rare, bred true for *S. alba* and 90 % of the inoculations were successful.

The host plants were inoculated at the 6–10-leaf stage. About 10^6 haploid sporidia of two stocks of opposite mating-type were mixed together in a small drop of water on a slide or watch glass. The mixture of sporidia was scraped off the slide with a mounted needle and smeared into wounds in the axils of the young leaves of the seedling. Diploid strains were inoculated as pure cultures using a similar procedure. The plants were allowed to flower naturally and at this stage the infected plants could be recognized by the swollen flower buds, split calyx, and purple spore-filled anthers (Baker, 1947). Infected buds were removed from the plants before they opened, and were stored in small capped bottles containing 1 cm depth of dried, sterile silica gel (B.D.H. 30–120 mesh). Brandspores harvested in this way remain viable for at least 3 years.

All crosses were analysed by sampling random sporidia produced from many meiotic divisions using the method of Holliday (1961*a*). About three infected

anthers were suspended in 9 ml sterile water in a Macartney bottle. The anthers were crushed with a flame-sterilized rod, 0·05 ml of this dense suspension of brandspores was spread over the plate of complete medium, and the plates were incubated for 48 h. At the end of this period there were numerous tiny colonies, many of which were derived from the germination of a single brandspore. Each of these colonies was of mixed genotype as meiotic segregation occurs during the germination of each brandspore and produces four haploid sporidia which bud indefinitely. The sporidia derived from a brandspore cannot fuse on this medium, and therefore each brandspore colony consists of a mixture of four distinct clones (tetrad colony). A plate of complete medium containing several thousand of these small tetrad colonies was flooded with about 2 ml of sterile water to obtain a suspension of sporidia derived from many meiotic divisions. The concentration of this suspension was estimated, a suitable dilution made, and appropriate aliquots were spread on a series of plates of complete medium. Each of the colonies that grew on these plates was derived from the germination of a single cell and was therefore of pure genotype. Mixed colonies produced by the germination of two or more cells at one site were rare (less than 0·5 %).

It is possible to classify the genotypes of these colonies directly by replicating from this complete medium to a series of test media (Holliday, 1961*a*). However, it was found more efficient to transfer the monosporidial colonies and to arrange them in rows on a fresh plate of complete medium (master plate). This master plate was then replicated to the appropriate test media, as in the haploidization experiments. The genotype of each colony was deduced from its growth on the test media.

(iv) *Mitotic crossing-over*

Spontaneous diploid segregants were very rare. Many diploid segregants were obtained when a diploid was irradiated with u.v. using a technique identical to that used to obtain mutants in haploid strains (Day & Jones, 1968).

3. RESULTS

The requirements of the 42 isolates used in the genetical analysis are summarized in Table 1 (see also Day & Jones, 1968). Mutants with similar requirements were classified by complementation tests before they were mapped by mitotic haploidization and by meiotic segregation.

(i) *Complementation tests*

The arginine, histidine, lysine, and methionine mutants were analysed by complementation tests. Some combinations of isolates were not possible as the mutants were not available in strains of opposite mating-type. All pairings of arginine mutants in different auxanographic groups complemented. Pairings of the same isolate did not complement, and the absence of complementation indicated that the three mutants in group 4 are allelic. Mutants C4 and C6 in group 3 were known to

Table 1. *Mutants used in the genetical analysis*

Mutant responds to:	Isolate no.	Gene symbol	Remarks
(1) Adenine, guanine, inosinic acid	31	ad_2	—
(2) NH_4^+, asparagine, arginine	125	*nir*	Uncertain whether or not these isolates are allelic
	126	*nir*	
(3) Arginine	C4	arg_1	Accumulates argininosuccinic acid
	C6	arg_2	Accumulates citrulline
	7161	arg_3	Accumulates ornithine
(4) Arginine, citrulline	114	cit_1	—
	115		
	143		
(5) Arginine, citrulline, ornithine	15	orn_1	These isolates may be complementary alleles
	32	orn_2	
(6) Arginine, citrulline, ornithine, glutamic acid, proline	121	pro_1	—
(7) Histidine	C2	his_1	—
	144		
	199		
	151	his_2	—
	186	his_2	—
	112	his_3	—
	113	his_4	—
(8) Inositol	729	$inos_1$	—
(9) Lysine	123	lys_1	—
	716	lys_2	—
	33		
	124		
	C3	lys_3	—
	83		
	84		
	184		
	191		
	195		
	197		
(10) Methionine	75	met_1	—
	145	met_1	—
(11) Methionine, cysteine, $S_2O_3^{2-}$	110	met_2	—
	183	?	183, 185 not yet classified by complementation tests
	185	?	
(12) Methionine, cysteine, $S_2O_3^{2-}$, SO_3^{2-}	C1	met_3	—
	7291		
	194		
(13) Pyridoxin	182	pdx_1	—
(14) SO_3^{2-}	C5	sul_1	—
	No requirements		
— Yellow colonies	C	*y*	Bright yellow colonies (recessive to normal pink colonies)

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be in different genes since they accumulate arginino-succinic acid and citrulline respectively under conditions of limiting arginine (J. Evans, A. W. Day & J. K. Jones, unpublished), and are described as arg_1 and arg_2 respectively. It was difficult to locate 7161 in the biosynthetic pathway since it appeared to accumulate ornithine but did not respond to citrulline. It complemented with both arg_1 and arg_2 and also with the citrulline mutants in group 4, and in the absence of further evidence is described as arg_3. The nine arginine mutants have therefore been classified into seven genes as shown in Table 1. Similarly, the histidine mutants were classified into four genes, and the lysine mutants into three genes. The methionine mutants were classified into at least three genes. Sufficient data was not obtained to classify isolates 183 and 185 and these isolates may therefore be alleles at the met_2 locus, or may represent 1 or 2 different methionine genes.

4. HAPLOIDIZATION

As haploidization proceeds independently of crossing-over and rarely coincides with it (Pontecorvo, 1956; Pontecorvo & Käfer, 1958), analysis of the haploid genotypes provides the most efficient method of assigning genes to linkage groups. During haploidization one member of each chromosome pair is lost, probably by non-disjunction (Käfer, 1961) and the process results in the random reassortment of whole chromosomes. Genes on the same chromosome remain linked, whereas genes on different chromosomes recombine randomly, and so it is possible to assign linkage groups unequivocally by examination for recombination between markers. Spontaneous haploidization was very rare: only one case was observed during the analysis of about 250 diploids. DL-*p*-fluorophenylalanine (PFP) is known to induce haploidization in *Aspergillus* (Morpurgo, in Lhoas, 1961) and was found to induce the same process in *U. violacea* (Day & Jones, 1968). About 200–500 papillae were produced on each plate of PFP medium. The rarity of spontaneous haploidization and the compact non-spreading growth of the sporidial colonies ensures that each haploid papillum is derived from a separate haploidization event. The mechanism of PFP-induced haploidization will be discussed in more detail in a later paper.

Approximately 500 diploid strains of various genotypes were synthesized and about 250 of these were used in the haploidization experiments. Some diploids were haploidized on PFP medium several times. Representative examples of the type of results obtained are described below, and the linkage data is summarized in Table 3.

Linkage was not found until many experiments had been analysed and most of the diploids showed segregation ratios which clearly demonstrated recombination for all the markers used. The haploid genotypes should be recovered in equal numbers, but abnormal segregation ratios were obtained occasionally. The results are therefore discussed under three headings: (*a*) results showing no linkage and with normal segregation; (*b*) results showing no linkage but with abnormal segregation; (*c*) results showing linkage.

Table 2. *Haploidization results showing no linkage and with normal segregation*

Diploid no.	Diploid genotype*	Genotypes of haploid segregants recovered†				Allele frequency	Recombination	Remarks
E19	32 124 *orn*$_2$ + a$_1$ + *lys*$_2$ a$_2$			+	8	o = 47 %	o-l = 47 %	The two marker genes in this diploid are on two separate chromosomes
				o	8	l = 50 %		
				l	9			
				o l	7			
					32			
GA	C C2 15 729 *y* *his*$_1$ + + a$_1$ + + *orn*$_1$ *inos*$_1$ a$_2$	y +	32	+	41‡	y = 65 %	y-i = 46 %	A small sample of papillae were tested for mating type. This gene also recombined randomly with the other genes. The 5 genes in this diploid are therefore on 5 separate chromosomes
		y h	35	h	10	o = 43 %	y-i = 56 %	
		y o	22	o	12	h = 46 %	h-i = 53 %	
		y i	25	i	7	i = 40 %	y-o = 52 %	
		y h o	27	h o	13		h-o = 56 %	
		y o i	25	o i	8		o-i = 43 %	
		y h i	19	h i	10			
		y h o i	21	h o i	9			
			206		110			
			Total 316					
GB	C C1 15 729 *y* *met*$_3$ + + a$_1$ + + *orn*$_1$ *inos*$_1$ a$_2$	y +	5	y m o	5	y = 100 %	y-m = 42 %	Only yellow papillae scored. All the 4 marker genes recombined randomly and are therefore on 4 separate chromosomes
		y m	8	y o i	4	m = 58 %	y-i = 48 %	
		y o	7	y m i	8	o = 48 %	y-o = 48 %	
		y i	4	y m o i	7	i = 48 %	m-o = 44 %	
							m-i = 56 %	
							o-i = 50 %	
			Total 48					
GZF	C C2 729 124 *y* *his*$_1$ + + a$_1$ + + *inos*$_1$ *lys*$_2$ a$_2$	y +	7	y h i	4	y = 100 %	y-h = 56 %	
		y h	4	y h l	5	h = 44 %	y-i = 47 %	
		y l	5	y i l	6	l = 51 %	y-l = 51 %	
		y i	7	y h i l	7	i = 53 %	h-i = 49 %	
							h-l = 58 %	
							i-l = 53 %	
			Total 45					

* The top line represents the genotype of the first haploid parent, and the bottom line that of the second parent. The number of the isolate used is shown above each gene symbol (see Table 1).

† The genotypes of the haploid segregants are described only by the recessive characters which they express, and an abbreviated form of the gene symbol is used.

‡ Many of these were not haploid, but appeared to be either diploid or aneuploid.

In many cases diploids heterozygous for the recessive yellow colony mutant were used. These diploids produced both pink and yellow haploid papillae, but it was not easy to distinguish haploid papillae from papillae that were not fully haploid. Consequently the data for many of the earlier experiments are derived mainly or wholly from the yellow papillae, which were known to be monosomic for one particular chromosome and were probably fully haploid. After sufficient experience it was possible to select pink haploid papillae by their colour which is paler than that of diploid or aneuploid papillae.

It was not convenient to classify large numbers of papillae for mating-type, although this is a useful marker gene, and the linkage data for this locus are therefore based on the analysis of few colonies. Since markers either recombine randomly or remain linked, haploidization experiments have the advantage that precise linkage data can be obtained from the analysis of very few colonies, and consequently the conclusions drawn from these results were considered to be accurate.

(*a*) *Results showing no linkage and with normal segregation*

The results of five diploids are shown in Table 2. All the haploid phenotypes that were possible theoretically were recovered from each diploid, and each gene recombined randomly with the other genes in that diploid. The allele frequencies were about 50% as expected, and the different phenotypes were recovered in approximately equal numbers, e.g. all the 16 possible phenotypes were recovered in more or less equal numbers from diploid GA.

(*b*) *Results showing no linkage but with abnormal segregation*

About 50% of the diploids analysed segregated normally and gave equal numbers of the different phenotypes. In about 15% of the diploids, one or two markers appeared with a low allele frequency (10–30%). This did not seriously affect the genetical analysis, as there were always sufficient segregants to establish the linkage relationships. Diploids E18 (below) and E4 (next section) provide examples of this type of segregation.

Diploid E18

Diploid genotype	Haploid segregants recovered		Allele frequencies	Recombination
124 110	+	29	l = 13%	l-m = 70%
$\frac{ys_2}{+}$ $\frac{+}{met^2}$ $\frac{a_1}{a_2}$	l	2	m = 34%	
	m	12		
	m l	4		

It is clear that the genes lys_2 and met_2 are unlinked as recombinants were obtained, but the allele frequencies of these genes were as low as 13% and 34%, respectively. These low frequencies were caused mainly by an excess of wild-type segregants. One of the prinicpal reasons for this excess was that many of the wild-type papillae were not haploid, but had large- or intermediate-sized cells and were probably

aneuploid. Only about 1 % of yellow papillae were aneuploid, and few of the papillae which expressed one or more of the nutritional markers had larger cells than the haploid stocks.

A complete genetical analysis was difficult in about 35 % of the diploids, as certain markers were either absent or rare (0–5 % allele frequencies) in the haploid progeny although the other markers segregated normally. These markers failed consistently to segregate from all the diploids in which they were present, and were therefore designated 'missing-markers'. It was not possible to determine their linkage relationships by the analysis of papillae, and they constituted such a large fraction of the markers (30 %) that their failure to segregate hindered the analysis of linkage groups and the determination of the minimum chromosome number. The cause of this missing-marker phenomenon was investigated and is reported in detail in a later paper. It was found that these markers failed to segregate because they were on chromosomes which became monosomic after PFP treatment. A modified technique was devised to isolate monosomics expressing the missing-markers, by streaking out the disomic papillae on complete medium, and it was then possible to map these markers. The results of this mapping will be discussed in more detail in a later paper, but are included in the summary of linkage relationships (Table 3).

(c) *Results showing linkage*

(1) *Diploid JE 1*

	C	C6	15	729	
Genotype ...	$\frac{y}{+}$	$\frac{arg_2}{+}$	$\frac{+}{orn_1}$	$\frac{+}{inos_1}$	$\frac{a_1}{a_2}$

Haploid segregants

Yellow colonies		Pink colonies	
y +	0	+	0
y i	0	i	0
y a	20	a	10
y o	5	o	1
y a i	6	a i	4
y o i	4	o i	2
	35		17

Total 52

Allele frequencies ... y = 67 % i = 31 % 'a + o' = 100 %

Recombination

y-a, y-o } = c. 50 % (estimate) a-i, o-i } = 50 % (est.)

y-i = 40 % a-o = c. 0 % (est.)

Haploids mutant for arg_2 were not distinguished from those mutant for both orn_1 and arg_2 and the 16 possible genotypes produced by random segregation of the four marker genes in this diploid consequently fall into 12 phenotypic classes. Eight phenotypes were found and all expressed either arg_2 or orn_1: none were

wild type with respect to these two genes. This result provided good evidence that arg_2 and orn_1 are linked. The other two genes, y and $inos_1$ recombined randomly, verifying the previous conclusion that they are not linked.

(2) *Diploid E 36*

	C	C2	729	15	32	
Genotype ...	$\frac{y}{+}$	$\frac{his_1}{+}$	$\frac{inos_1}{+}$	$\frac{orn_1}{+}$	$\frac{+}{orn_2}$	$\frac{a_2}{a_1}$

Haploid segregants

Yellow papillae		Pink papillae		
y +	0	+	0	
y i	0	i	0	
y h	0	h	0	
y 'o'	4	'o'	6	
y i h	0	i h	0	
y 'o' h	4	'o' h	1	
y 'o' i	6	'o' i	3	
y 'o' i h	7	'o' i h	1	
	21		11	Total 32

'o' = $orn_1 + orn_2$ (indistinguishable)

Allele frequencies ... y = 66 % i = 53 % h = 41 % 'o' = 100 %

Recombination

$y\text{-}o_1$, $y\text{-}o_2$, $o_1\text{-}i$, $o_1\text{-}h$, $o_2\text{-}i$, $o_2\text{-}h$ } *c.* 50 % (est.)

y-h = 38 %
y-i = 38 %
h-i = 44 %
$o_1\text{-}o_2$ = *c.* 0 % (est.)

Orn_1 was not distinguished from orn_2 and the 32 possible genotypes therefore fall into 16 phenotypic classs. Eight classes were found, all of which expressed a requirement for ornithine. It is apparent therefore that orn_1 and orn_2 were linked in repulsion in this diploid, and that the other genes y, his_1 and $inos_1$ recombine randomly as they did in previous experiments. Orn_1 and orn_2 complement and may be either distinct genes, or complementary alleles of the same gene.

(3) *Diploid B 30*

In the analysis, shown next page, haploids carrying arg_3 were not distinguished from those carrying both arg_3 and orn_1. Theoretically the 64 genoytpes produced by independent assortment would therefore fall into 48 phenotypic classes, but only 32 classes were found, all of which were mutant for either arg_3 or orn_1, and none were wild type for both the genes. Thus arg_3 and orn_1 are linked, but the other genes reassort randomly.

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	C	729	C1	15	7161	716	
Genotype ...	$\frac{y}{+}$	$\frac{inos_1}{+}$	$\frac{met_3}{+}$	$\frac{orn_1}{+}$	$\frac{+}{arg_3}$	$\frac{+}{lys_2}$	$\frac{a_1}{a_2}$

Haploid segregants

Yellow papillae		Pink papillae		
y o	9	o	4	
y a	9	a	5	
y o i	3	o i	1	
y a i	2	a i	0	
y o m	7	o m	0	
y a m	3	a m	1	
y o l	3	o l	1	
y a l	3	a l	1	
y o i l	4	o i l	0	
y a i l	2	a i l	0	
y o m l	6	o m l	1	
y a m l	6	a m l	1	
y o i m	5	o i m	0	
y a i m	4	a i m	0	
y o i m l	1	o i m l	0	
y a i m l	1	a i m l	0	
	68		15	Total 83

Allele frequencies

y = 82 % o = 54 % m = 43 %
a = 46 % l = 36 % i = 28 %

Recombination

y-i = 57 % i-m = 45 %
y-m = 45 % i-l = 55 %
y-l = 45 % m-l = 59 %
o-a = *c.* 0 % (est.)

y-o, y-a, i-o, i-a } *c.* 50 % (est.) { m-o, m-a, o-l, a-l

The results from these three diploids, JE 1, E 36, and B 30 show that four genes concerned with arginine biosynthesis, arg_2, arg_3, orn_1 and orn_2, are on the same chromosome.

(4) *Diploid H 7*

	84	C2	123	
Genotype ...	$\frac{lys_3}{+}$	$\frac{his_1}{+}$	$\frac{+}{lys_1}$	$\frac{a_1}{a_2}$

Haploid segrants

+	0
'l'	38
h	12
'l' h	3
	53

'l' = either lys_1 or lys_3 or both

Allele frequencies ... h = 28 % 'l' = 77 %

Recombination

l_1-h = *c.* 0 % (est.)

l_3-h, l_1-l_3 } *c.* 50 % (est.)

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The absence of haploid wild types indicates linkage between pairs of recessive alleles in repulsion, i.e. between lys_1 and either lys_3 or his_1. As papillae which expressed his_1 but which had no requirement for lysine were found, lys_1 and his_1 must be linked. The deficiency of '1' h types could be due to selection, as these types need a specific lysine : histidine balance in the medium, but it is more likely to be caused by the non-segregation of the lys_3 marker which was one of the missing-markers.

(5) *Diploid E* 4

	C	15	729	C2	110	
Genotype ...	*y* / +	*orn*$_1$ / +	*inos*$_1$ / +	*his*$_1$ / +	+ / *met*$_2$	a / a$_1$

Haploid segregants

Yellow colonies		Pink colonies		
y m	13	+	0	
y m o	10	m	29	
y m i	18	m o	6	
y m o i	17	m i	4	
y h	2	m o i	7	
y h o	2	h	3	
y h i	1	h o	1	
y h o i	2	h i	2	
		h o i	1	
	65		53	Total 118

Allele frequencies ... y = 55 % o = 39 % m = 88 % h = 12 %

Recombination

y-m = 55 %	y-h = 55 %	o-h = 41 %
y-o = 42 %	m-h = 0 %	o-m = 41 %

Theoretically, if the five marker genes in diploid E 4 are not linked, there are 32 possible haploid genotypes. Only 16 were observed, and as no wild types were found the linkage must have been between genes in repulsion in the diploid, i.e. between met_2 and one of the other genes. No recombinants were found for the genes met_2 and his_1, and therefore these were linked. The other genes segregated independently as previously. As it has already been shown that his_1 is linked to lys_1 (diploid H 7), the three genes met_2, lys_1, and his_1 must be on the same chromosome. The results from this diploid also illustrate some of the aberrancies that were observed occasionally, e.g. the excess of *m* parental types and the low allele frequency of his_1.

Table 3. *Summary of the linkage data obtained by analysis of mitotic haploidization and meiotic crossing-over in* Ustilago violacea

orn_2	cit_1	arg_1	arg_2	arg_3	pro_1	ad_2	his_1	his_2	his_3	his_4	pdx_1	$inos_1$	lys_1	lys_2	lys_3	met_1	met_2	met_3	sul_1	nir_1	nir_2	y	mt	Gene symbol
L	0	0	L	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	
–	+	+	10	6	–	–	+	+	–	+	–	+	–	+	+	+	–	+	–	–	–	+	–	orn_1
	–	–	–	–	–	–	–	–	–	–	–	0	0	0	0	–	–	–	–	–	–	–	–	
	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	orn_2
		0	–	–	L	–	0	–	–	–	0	0	–	0	L	L	–	–	–	–	–	0	–	
		–	–	–	30	–	–	–	–	–	–	+	–	+	44	2·5	–	–	–	–	–	+	–	cit_1
			–	–	0	–	0	–	–	–	–	0	0	–	0	0	–	–	–	–	–	0	0	
				–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	+	–	arg_1
				–	–	0	–	0	–	–	–	0	–	0	–	–	–	–	–	–	–	0	–	
					–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	arg_2
					–	0	–	–	0	–	–	0	–	0	0	0	0	0	–	–	–	0	0	
					–	–	+	–	–	–	–	+	–	+	–	–	–	+	–	–	–	+	–	arg_3
						–	0	–	0	–	–	0	–	–	–	L	–	–	–	–	–	0	–	
						–	–	–	–	–	–	–	–	+	15	19	–	–	–	–	–	+	–	pro_1
							–	–	–	–	–	0	–	0	–	–	–	0	–	–	–	0	–	
							3·3	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	ad_2
								–	–	–	0	0	L	0	0	0	L	0	–	–	–	0	0	
								–	+	+	–	+	41	+	+	+	–	+	–	–	–	+	–	his_1
									–	–	–	0	–	–	0	0	–	0	0	–	–	0	0	
									–	+	–	+	–	+	–	+	–	–	–	–	–	–	–	his_2
										–	–	0	–	0	0	0	–	0	–	–	–	0	0	
										–	–	–	–	–	–	–	–	–	–	–	–	+	–	his_3
											0	0	–	0	0	0	–	0	–	–	–	0	0	
											–	+	+	+	+	+	–	+	–	–	–	+	–	his_4
												0	0	–	0	0	–	–	–	–	–	–	0	
												–	–	–	–	–	–	–	–	–	–	–	–	pdx_1
													–	0	0	0	0	0	0	0	0	0	0	
													+	+	+	+	–	+	+	–	–	+	–	$inos_1$
														0	0	0	–	–	–	–	–	0	–	
														+	+	+	–	+	–	–	–	+	–	lys_1
															0	0	0	0	–	–	–	0	0	
															+	+	–	+	–	–	+	+	–	lys_2
																L	–	0	–	–	–	0	0	
																+	–	–	–	–	–	+	–	lys_3
																	0	0	–	–	–	0	0	
																	+	+	–	–	–	+	–	met_1
																		0	0	–	–	0	0	
																		–	–	–	–	–	–	met_2
																			0	0	0	0	0	
																			–	–	–	+	–	met_3
																				–	–	0	–	
																				–	–	+	–	sul_1
																					–	0	0	
																					–	–	–	nir_1
																						0	0	
																						+	–	nir_2
																							0	
																							–	y

The letter on the upper line represents the result of analysis by mitotic haploidization. L = markers linked; 0 = markers not linked; – = no data. The symbol on the lower line represents the result of analysis by meiotic crossing-over. + = recombination frequency does not differ significantly from 50 % ($P = 0{\cdot}05$). 10 = recombination frequency (%). – = no data. Note that (1) orn_1 and orn_2, nir_1 and nir_2 have not been proved to be non-allelic. (2) cit_1, pro_1, lys_3, and met_1 behave as missing markers during the haploidization analysis.

5. SUMMARY OF THE LINKAGE GROUPS OBTAINED BY ANALYSIS OF MITOTIC HAPLOIDIZATION

Table 3 illustrates the linkage relationships of the mutants that were used in these studies. Multiply-marked stocks were not available until late in the project and consequently most diploids had few (2–5) markers. It was therefore necessary to analyse a great number of diploids before a mutant could be assigned to a new linkage group. The classification of the markers into linkage groups, based on the data in Table 3, is shown in Table 4.

Ten linkage groups were identified unequivocally and two other mutants must be tested with 2–3 more isolates only, to confirm their identity as distinct linkage groups. Of the ten established linkage groups, two (IX and X in Table 4) were associated with missing-markers, as these chromosomes tended to remain disomic after treatment with PFP. The other eight groups were associated with chromosomes that were haploidized regularly by PFP.

There was no doubt that most of the papillae produced from a diploid on PFP medium were haploid, or more correctly disomic for 1–2 chromosomes. Thus the segregants had small haploid-sized cells with a positive mating-type reaction quite distinct from the large cells of the diploid with their neutral mating-type reaction (Day & Jones, 1968). Also, the analysis of papillae from many diploids showed that a wide range of genotypes, both parental and recombinant, was obtained from each diploid. The types and range of genotype were characteristic of haploidization rather than mitotic crossing-over or non-disjunction, as these latter types of segregation do not usually produce phenotypes expressing recessive characters from both parents simultaneously (see Pontecorvo and Käfer, 1958).

(*i*) *Meiotic segregation*

As the haploidization results were not available until relatively late in the project, it was not possible to make many crosses between markers that had been shown to be linked by this method. Most crosses were between mutants selected at random and which were later shown to be unlinked from studies of haploidization. The linkage data from the 123 crosses that were analysed is summarized in Table 3. Most crosses were between haploid mutants inoculated simultaneously into the host plant, but a few infections produced by the inoculation of a single diploid culture were analysed also.

(*a*) *Crosses involving unlinked markers*

The three crosses illustrated in Table 5 were typical of the type of result obtained, and show that all the possible phenotypes were recovered with approximately equal frequency. Low allele frequencies were obtained in a few crosses. These were caused either by an excess of wild-type sporidia or by a deficiency of the multiply auxotrophic classes. Both these effects, which were also reported for *Ustilago maydis* by Holliday (1961*a*), were presumably due to differences in the

Table 4. *The linkage groups obtained in* Ustilago violacea

									Missing-marker chromosomes			
Linkage groups ...	I	II †	III ‡	IV	V ‡	VI	VII	VIII	IX	X	XI †‡	XII ‡
Genes and isolates	arg_2	his_1	lys_2	$inos_1$	met_3	a_1/a_2	*y*	his_3	lys_3	his_2	his_4	arg_1
	C6	C2	716	729	11C1		C	112	C3	151	113	11C4
	arg_3	(144)	33	pdx_1	7291				84	(186)		
	7161	met_2	124	182	(194)				184			
	orn_1	110							195			
	15	lys_1							197			
	orn_2	123							(83)			
	32	ad_2							(191)			
		31*							met_1			
									75			
									145			
									cit_1			
									115			
									143			
									(114)			
									pro_1			
									121			

Notes: Markers in parentheses were not used in the analysis, but are included as the complementation tests showed that they were allelic with markers that were tested.

* Inclusion of ad_2 in group II based on tentative evidence from meiotic data.

† Tests distinguishing linkage groups II and XI have not been made.

‡ Tests distinguishing linkage group XII from linkage groups III, V, and XI have not been made.

4. Other markers which have been used to a lesser extent, and which have not been assigned to a linkage group by this method are: nir_1-M125, nir_2-M126 (missing markers), *sul*-M11C5, and ad_2-M31*.

Table 5. *The results of some crosses between unlinked markers*

Cross	Phenotype of segregants recovered: Yellow		Pink		Allele frequency	Recombination
(1) C C2 75	y+	23	+	16	y = 58 %	y-m = 53 %
P_1 *y his*$_1$ + a_1	y m	18	m	18	m = 49 %	m-h = 51 %
P_2 + + *met*$_1$ a_2	y h	23	h	22	h = 55 %	y-h = 52 %
	y m h	32	m h	14		
		Total 166				
(2) C C1 15 729	y +	66	+	61	y = 48 %	y-m = 50 %
P_1 y *met*$_3$ + + a_1	y m	50	m	53	m = 49 %	y-o = 57 %
P_2 + + *orn*$_1$ *inos*$_1$ a_2	y o	53	o	52	o = 50 %	y-i = 49 %
	y i	39	i	64	i = 50 %	m-i = 57 %
	y m o	43	m o	57		m-o = 52 %
	y m i	49	m i	53		o-i = 47 %
	y o i	55	o i	60		
	y m o i	63	m o i	60		
		418		460		
		Total 878				
(3) Diploid GY	All colonies pink					
729 729 17 16 1 716						
$\frac{inos_1}{+}$ $\frac{met_3}{+}$ $\frac{+}{arg_3}$ $\frac{+}{lys_2}$ $\frac{a_1}{a_2}$	+	22	i m	18	a = 46 %	a-l = 49 %
	a	9	i l	12	i = 50 %	a-i = 53 %
	i	9	m l	11	m = 45 %	a-m = 52 %
	m	6	a i m	12	l = 49 %	i-m = 44 %
	l	18	a i l	14		i-l = 48 %
	a i	14	a m l	10		m-l = 48 %
	a m	12	i m l	11		
	a l	12	a i m l	10		
		Total 200				

Table 6. *Crosses involving markers in linkage groups I and II*

Cross	Haploid segregants recovered: Yellow		Pink		Allele frequencies	Recombination
			Linkage group I			
(1) C C6 15 729 P_1 *y* arg_1 + + a_1 P_2 + + orn_1 $inos_1$ a_2	y +	21	+	14	y = 55 %	y-i = 53 %
	y i	18	i	15	i = 53 %	y-o, y-a, a-i, o-i } *c.* 50 %
	y 'a'	302	'a'	283	'a' = 95 %	
	y 'a' i	391	'a' i	276		
		732		588		a-o = 10·3 % (see text)
	Total 1320					
	'a' = either arg_2 or orn_1, or both					
(2) A reciprocal cross of (1) C C6 15 729 P_1 + + orn_1 + a_1 P^2 *y* arg_2 + $inos_1$ a_2	y +	5	+	3	y = 51 %	y-i = 55 %
	y i	1	i	0	i = 43 %	y-o, y-a, a-i, o-i } = *c.* 50 %
	y 'a'	42	'a'	51	'a' = 95 %	
	y 'a' i	43	'a' i	33		
		91		87		a-o = 10·3 % (see text)
	Total 178					
	'a' = either arg_2 or orn_1, or both					
(3) 729 15 7161 716 P_1 $inos_1$ orn_1 + + a_1 P_2 + + arg_3 lys_2 a_2			+	1	i = 46 %	l-i = 45 %
			i	2	l = 42 %	l-a, a-i, l-o, o-i } = *c.* 50 %
			l	0	'a' = 97 %	
			'a'	27		
			l i	0		
			'a' *i*	23		
			'a' l	29		
			'a' i l	17		a-o = 6 % (see text)
				99		
	'a' = either arg_3 or orn_1, or both					

Table 6 (*cont.*)

Cross	Haploid segregants recovered				Allele frequencies	Recombination
	Yellow		Pink			
	Linkage group II					
(4) C 123 C2	y +	35	+	31	y = 58 %	y-h = 47 %
P_1 + lys_1 + a_1	y l	54	l	40	h = 53 %	y-l = 49 %
P_2 y + his_1 a_2	y h	65	h	40	l = 49 %	l-h = 41 %
	y l h	42	l h	31		
		196		142		
	Total 338					
			+	7	h = 97 %	h-ad = 3·3 %
(5) 31 144			h	202	ad = 0 %	(see text)
P_2 ad_2 + a_1			ad	0		
P_2 + his_1 a_2			h ad	0		

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growth rate of sporidia with different requirements. Evidence for this was obtained by an experiment in which the tetrad colonies were left for 48, 96, or 120 h before they were sampled. The results are shown below.

	Cross		
	15	729	
	$(orn_1 + a_1) \times (+ inos_1\, a_2)$		
	Proportion of each phenotype		
Haploid segregants	After 48 h	96 h	120 h
+	24 %	39 %	84 %
o	26 %	27 %	9·3 %
i	24 %	22 %	9·2 %
o i	26 %	12 %	0·1 %
Total analysed	207	406	1968

The segregants were in equal frequencies when the tetrad colonies had grown for 48 h, but after 96 h there was an excess of wild types and a deficiency of the double mutant types which became more marked after 120 h. Thus, as the tetrad colonies grow there is increasing competition for the available nutrients, and clones with few nutrient requirements have a high selective advantage and bud more quickly. Such clones therefore tend to be represented more frequently than multiple auxotrophs amongst the random sporidia isolated from the tetrad colonies. In the experiments, therefore, the tetrad colonies were always sampled after 48 h to minimize selection by growth rate. Occasionally some markers-failed to appear amongst the haploid progeny of a cross. Unlike the missing markers in haploidization experiments this did not occur regularly for particular markers, and it could usually be explained by back mutation in one of the haploid parents.

(*b*) *Crosses between linked markers* (*Table 6*)

(1) *Linkage group I.* Haploidization studies had shown that both arginine mutants, arg_2 and arg_3, and the ornithine mutant orn_1 were linked. Since it was not necessary to distinguish arginine mutants from ornithine mutants for the determination of the recombination frequencies, ornithine was not included in the test series of media. Consequently the recombinant class *arg*, *orn* was indistinguishable from both parental classes, *arg* + and + *orn*, and one half of the recombinant classes only, i.e. the wild types, could be recognized. The recombination frequencies for these genes in the three crosses were calculated from the formula (2 × wild-type recombinants)/ total. It is possible that this may have given an overestimate as it had been found previously that prototrophs may tend to grow faster than auxotrophs on complete medium.

The estimates of the map distance arg_2–orn_1 obtained from crosses 1 and 2 were very similar at just over 10 %. The estimate from cross 1 was obtained from a large sample of random sporidia and is therefore likely to be accurate. The recombination frequency of about 6 % for arg_3 and orn_1 (cross 3) was obtained from a

Table 7. *The mapping of genes in linkage group IX*

	Cross	Haploid genotypes recovered	Allele frequencies	Recombination
(1)	143 121	Pink Yellow		
	P_1 cit_1 + a_1	++ 29	c = 51 %	c-p = 30 %
	P_2 + pro_1 a_2	c + 68	p = 48 %	
		+ p 63		
		c p 28		
		188		
(2)	143 C3			
	P_1 cit_1 + a_1	++ 59	c = 27 %	c-l = 43 %
	P_2 + lys_3 a_2	c + 30	l = 49 %	
		+ l 68		
		c l 16		
		173		
(3)	C 143 C3	++ 43 y ++ 42	y = 51 %	y-l = 53 %
	P_1 y + lys_2	a_1 c + 39 y c + 57	c = 48 %	y-c = 52 %
	P_2 + cit_1 +	a_2 + l 63 y + l 60	l = 54 %	c-l = 45 %
		c l 49 y c l 44		
		194 203		
(4)	143 145			
	P_1 cit_1 + a_1	++ 18	c = 50 %	c-m = 2·5 %
	P_2 + met_1 a_2	c + 656	m = 49 %	
		+m 645		
		c m 15		
		1334		
(5)	75 121	++ 51	m = 80 %	m-p = 19 %
	P_1 met_1 + a_1	m + 216	p = 0·7 %	(estimate
	P_2 + pro_1 a_2	+ p 2		from ++
		m p 0		and m+ types
		269		only)
(6)	C C3 145	++ 21 y ++ 19	y = 51 %	y-l = 53 %
	P_1 y lys_3 + a_1	l + 16 y l + 17	l = 39 %	y-m = 52 %
	P^2 + + met_1 a_2	+ m 25 y + m 33	m = 55 %	l-m = 44 %
		l m 18 y l m 14		
		80 83		
(7*a*)	C C3 121			
	P_1 y lys + a_1	+++ 15 y ++ 15	y = 51 %	y-l = 51 %
	P_2 + + pro_1 a_2	+ l +102 y l + 99	l = 56 %	y-p = 52 %
		++ p 70 y + p 79	p = 43 %	l-p = 14 %
		+ l p 12 y l p 14		
		199 207		
(7*b*) As 7*a* (Second sampling; yellow and pink colonies not distinguished)		++ = 12 l + = 64 + p = 2 l p = 0 78	l = 82 % p = 2·5 %	l-p = 16 % (estimate from ++ and l+ types only)

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small sample of random sporidia and therefore may be less accurate. The results from these three crosses confirm the result from haploidization studies that arg_2, arg_3, and orn_1 are linked, and show that the linkage is close. The other genes $inos_1$ and lys_2 segregated at random, as they did in other crosses and during haploidization.

(2) *Linkage group II*. The genes lys_1 and his_1 were shown to be linked during the analysis of mitotic haploidization. Cross 4 (Table 6) demonstrates that they are relatively far apart (41 cross-over units) and confirms that neither is linked to the *y* gene.

In cross 5 (Table 6) the ad_2 marker failed to segregate. It is unlikely that this was due to loss of ad_2 marker in the haploid parental stock before the cross was made, as the allele frequency for his_1 was 97 % and not 50 % as would then have been expected. The simplest explanation is probably that ad_2 was lethal during the germination of the brandspores in this cross, so that only half of the progeny was recovered, i.e. those lacking the ad_2 chromosome. If so, an estimate of linkage can be made using the wild-type recombinants, and this indicates that his_1 and ad_2 are 3·3 units apart. Little data is available on the linkage relationships of ad_2 with other genes. The segregation of pro_1 in crosses 7*a* and 7*b* (Table 7) provided evidence that this interpretation for the non-segregation of ad_2 is the correct one.

(3) *Linkage group IX*. Most of the missing-markers were in linkage group IX, and conseqently linkage relationships in this group have been analysed more completely. The results of several crosses are presented in Table 7.

The four genes known to be in linkage group IX, met_1, pro_1, cit_1, and lys_3, were crossed in pairs in all possible combinations. The results fully confirmed the evidence from mitotic haploidization that they were linked. Three crosses (2, 5 and 7*a*) gave unusual segregations but the linkage data obtained from these did not oppose that derived from the other crosses. In cross 2 ($cit_1 \times lys_3$) there was an excess of wild types and a deficiency of diauxotrophs, but the recombination frequency obtained agreed closely with that obtained from the reciprocal cross (cross 3) in which the segregation was normal. In crosses 5 and 7*b* the marker pro_1 rarely appeared amongst the haploid progeny, although it segregated normally in crosses 1 and 7*a*. This proline marker did not segregate in several other crosses involving unlinked genes, and it appears that in certain environmental conditions it is lethal during germination of the brandspore. The environmental conditions that cause this lethality are not known, and it is uncertain whether the effect is on the production and development of the promycelium or on the budding of the sporidia.

Crosses 7*a* and 7*b* show that this lethal effect does not disturb the ratio of segregants with the $+^{pro}$ allele, and that recombination frequencies can be analysed from the remaining genotypes. The frequency of recombination between lys_3 and pro_1 was very similar in the two samples 7*a* and 7*b* of the same cross. It can therefore be assumed that the estimates of linkage between pro_1 and met_1 (cross 5) and between ad_2 and his_1 (discussed previously) are reasonably acurate.

(c) *Summary of the linkage data obtained from analysis of meiotic segregation ratios*

Table 3 summarizes the linkage results that were obtained from the analysis of meiotic segregation in 123 crosses. These results should be compared with those obtained by mitotic haploidization. Note that the two sets of data do not conflict. Preliminary linkage maps of linkage groups I, II and IX using the data in Tables 6 and 7 are illustrated in Fig. 1.

(ii) *Mitotic crossing-over*

Analysis of mitotic crossing-over in diploids containing linked markers in coupling has been widely used in several species of fungi, e.g. *Aspergillus nidulans* (Pontecorvo & Käfer, 1958) and *Ustilago maydis* (Holliday, 1961*b*). The technique is useful for mapping the relative position of markers and centromeres, since after mitotic crossing-over the whole chromosome arm distal to the point of cross-over may become homozygous.

One suitable diploid (G3) containing the linked genes lys_1 and his_1 (linkage group II) was synthesized and induced to segregate by irradiation with u.v.

Diploid G3

	C	123 C2	C4	75
Genotype ...	$\frac{+}{y}$	$\frac{lys_1\ his_1}{+\quad +}$	$\frac{+}{arg_1}$	$\frac{+}{met_1}$ $\frac{a_1}{a_2}$

180 sec of irradiation were given and 6,150 surviving colonies were scored.

	% of survivors
Yellow prototrophic colonies ...	21 = 0·34
Pink auxotrophic colonies:	
Homozygous for:	
arg_1	9 = 0·14
met_1	6 = 0·10
$arg_1 + met_1$	1* = 0·02
lys_1	6 = 0·10
his_1	7 = 0·11
$lys_1 + his_1$	2 = 0·03
	19 = 0·31

* Sporidia of intermediate size—probably aneuploid.

After mitotic crossing-over, homozygosis of a proximal locus is accompanied automatically by homozygosis of a distal locus. Therefore, since the linked genes lys_1 and his_1 became homozygous independently and equally frequently, they are probably in different arms of the same chromosome with the centromere approximately midway between them. The two segregants requiring both lysine and histidine could have been caused either by simultaneous crossing-over in both arms of the chromosome, or by non-disjunction (Pontecorvo & Käfer, 1958). The other genes, *y*, arg_1 and met_1, became homozygous independently, which supports the evidence from studies of haploidization that they are on separate chromosomes.

6. DISCUSSION

(i) *Techniques*

The complementation test has been used widely in genetic studies of many fungi (Fincham, 1966). Growth of the heterokaryon has been the criterion for complementation in most filamentous fungi (Fincham & Day, 1965) but it is possible to compare complementation in diploids and heterokaryons of *Aspergillus* species (Pontecorvo, 1963). The phenotype of diploids is used as the basis of the complementation test in yeasts (e.g. Roman, 1956) and in *U. maydis* (Dr R. Holliday, personal communication) and was also found to be feasible in *U. violacea*. The translucent appearance of the diploid colonies of *U. violacea* could be distinguished easily from the more opaque haploid colonies, and the diploid complementation test was convenient for determining whether or not mutants with similar nutritional requirements were allelic.

The classification of the arginine, histidine, lysine and methionine genes obtained by complementation analysis has been fully supported by the analysis of mitotic and meiotic recombination. No mutants were classified as allelic which were later shown to recombine frequently during mitotic or meiotic segregation. No intragenic complementation was observed, although a group of 7 allelic lysine mutants were studied. This is not surprising, as the number of mutants that do show complementation with others in the same gene is often small (Fincham, 1966), and therefore tests of allelism based on complementation are usually efficient.

Haploidization has overwhelming advantages as a technique for assigning new markers to linkage groups (McCully & Forbes, 1965; Roper, 1966). Roper (1966) has reviewed the species of fungi in which the parasexual cycle has been described. The most thorough investigation of this cycle has been with the sexual species *Aspergillus nidulans*, and here the segregants may be explained by crossing-over, non-disjunction or haploidization. However, parasexual segregation in some other fungi appears to differ from that observed in *A. nidulans* (Roper, 1966). In some fungi, e.g. *A. fumigatus* (Strømnaes & Garber, 1963), the haploidization data were not consistent and an analysis of linkage was not possible. This was due mainly to a wide variation in the frequency of haploid genotypes, some types not being recovered at all. This problem was not encountered in *U. violacea* and the linkage data were always consistent and easy to interpret. Consequently it was possible to make a detailed genetical analysis of the species by a study of segregation during mitotic haploidization. It is interesting that PFP does not induce haploidization in the related species *U. maydis* even though the same method has been tried (Dr R. Holliday, personal communication). Mitotic haploidization in a wide range of fungi is reviewed in a later paper.

Although several diploids of *U. violacea* which contained genes from 2·5–41 meiotic units apart were haploidized, no haploid segregant recombinant for these genes was recovered. Thus in *U. violacea* as in *A. nidulans* (Pontecorvo & Käfer, 1958) mitotic crossing-over and haploidization are independent processes which rarely coincide. Consequently genes on the same chromosome remain linked during

haploidization, whereas genes on different chromosomes recombine randomly. In both these species of fungi genetical analysis via the parasexual cycle supports and confirms analysis of meiotic segregation. Hence the studies of haploidization indicating that there are at least 10–12 linkage groups provides strong evidence that the haploid chromosome number is greater than 10–12.

One possible disadvantage of *U. violacea* in genetical experiments is the duration of the sexual cycle. This took 3–4 months but could be shortened to about 1 month by the inoculation of the flowers of a mature plant in artificially lighted greenhouses (Baker, 1947). The purity of the seed used is also a very important factor. Care is needed to obtain seed of *Silene alba* uncontaminated by genes from *S. dioica*, as the two species are known to hybridize frequently (Baker, 1948). The most reliable method therefore is to collect seed from typical *S. alba* plants growing in places where *S. dioica* is rare, and to produce seed by controlling pollinations.

It would be very useful if brandspores could be induced to form in culture. Although most species of smut fungi grow only as haploid sporidia in culture, the other stages of the life-cycle being obligately parastic, a few species have been reported which can complete their life-cycle in culture if given suitable conditions (review in Fischer & Holton, 1957). However, in most of these reports the brandspores formed in culture failed to germinate and there is doubt whether or not they were diploid. Some reports of diploid brandspores of *Tilletia caries* and *T. controversa* formed in culture have been confirmed (e.g. Trione, 1964), and it is possible that in the correct cultural conditions all species in the Ustilaginales could complete their life-cycle on an artificial medium. Vigorous dikaryotic mycelium and diploid brandspores of *U. violacea* have not been produced in culture. An attempt to induce diploid sporidia to develop into brandspores or divide meiotically by using growth regulators such as indole acetic acid, gibberellic acid, and kinin was unsuccessful. Extracts of *Silene alba* leaves and flowers also did not affect the development of diploid sporidia.

(ii) *Linkage data*

(*a*) *The chromosome number*

The analysis of haploidization in many diploids demonstrated the existence of at least 10 and probably 12 linkage groups, and therefore of at least 10–12 chromosomes in the haploid sporidia. Since only a small sample of 21 genes were mapped, and six of the linkage groups contained a single marker only (Table 3) it seems likely that the haploid chromosome number is much greater than 10–12. This result is in conflict with the observations of several cytologists. Harper (1898) reported a haploid chromosome number of 8–10 for *Ustilago scabiosae*, a species which Kniep (1926) showed to be closely related to *U. violacea*, but most cytologists since then have concluded that the haploid chromosome number of several species of *Ustilago* is 2 (Kharbush, 1927, 1928; D. T. Wang, 1932, 1934; C. S. Wang, 1943; Huttig, 1933; Hirschhorn, 1945; Das, 1949; Fischer & Holton, 1957; Person & Wighton, 1964). D. T. Wang, (1934) included observations of *U. violacea* which was reported to have two chromosomes. The deduction from the haploidization

data that n is greater than 10–12 is supported by the meiotic data. There was no evidence that crossing-over during haploidization was producing an overestimate of the number of chromosomes.

In view of the discrepancy between the present genetical data and previous cytological observations, an attempt was made to count the number of chromosomes in haploid sporidia. The results of this investigation will be published in detail elsewhere, but preliminary observations indicate that the haploid sporidia contain many more than two chromosomes. Genetical evidence from *U. maydis*, although incomplete, suggests that there may be at least five chromosome arms in this species, and consequently more than two chromosomes (Holliday, 1964). The results of crosses in *U. hordei* (Dr C. H. Hood, personal communication) again suggest a chromosome number greater than 2. Therefore it seems highly probable that previous reports that $n = 2$ in *Ustilago* species are incorrect and that *U. violacea*, and possibly other species of *Ustilago*, have a haploid chromosome number higher than 10. There is a similar discrepancy between genetical and cytological evidence in *Saccharomyces cerevisiae*. Mortimer & Hawthorne (1966) reported at least 15 linkage groups, but no satisfactory preparations of the budding phase have been reported and the preparations of meiotic cells do not show the expected differences in the number of chromosomes in cells of different ploidy (Robinow & Bakerspiegel, 1965, and personal communication.)

(*b*) *Chromosome maps*

All crosses were analysed by the isolation of random products of meiosis. Deviations from normal segregation occurred in some crosses in *U. violacea* but were rarely large enough to make the analysis of linkage difficult. The results obtained from analysis of the random products of meiosis were used to form linkage maps for three chromosomes.

(1) *Linkage group I*. This linkage group was shown by analysis of mitotic haploidization to contain four genes concerned with the biosynthesis of arginine: arg_2, arg_3, orn_1 and orn_2. The meiotic segregation ratios (Table 6) indicated that arg_2 is about ten cross-over units away from orn_1, and that this latter gene is about six units from arg_3. Thus arg_3 and arg_2 may be either four or 16 units apart (see Fig. 1*a*). No information was available on the position of orn_2, and it has not been proven that this gene is non-allelic to orn_1.

(2) *Linkage group II*. The analysis of mitotic haploidization indicated that this linkage group contained three markers, lys_1, his_1 and met_2. Only one suitable cross was available for analysis and this indicated that lys_1 and his_1 were relatively far apart, having a recombination frequency of 41 % (Table 6). The data from the analysis of u.v.-induced diploid segregants from a diploid with these genes in coupling indicated that the centromere was located approximately midway between lys_2 and his_1. A second cross demonstrated that ad_2, which had not been located by the haploidization method, was closely linked to his_1. The recombination frequency was estimated to be 3·3 %, although the segregation ratios were aberrant

as the ad_2 marker did not segregate. Thus this chromosome may be represented tentatively as in Fig. 1*b*.

(3) *Linkage group IX*. At least four markers were known to be in linkage group IX, each of which behaved as a missing-marker during haploidization. The recombination values obtained during meiotic segregation (Table 7) were used to form a map of the chromosome (Fig. 1*c*). No data on the position of the centromere is available. The map of linkage group IX shown in Fig. 1*c* is consistent and free of important anomalies, although the frequency of recombination between pro_1 and

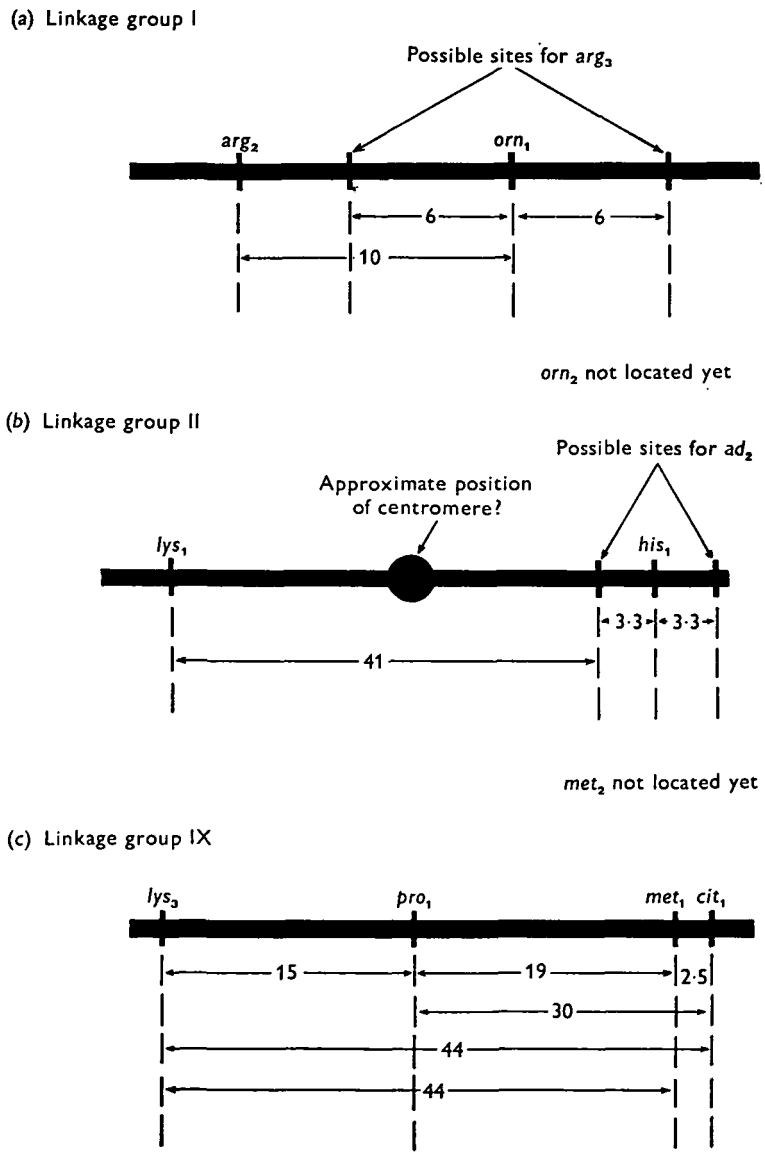

Fig. 1. Linkage maps of chromosomes I, II and IX.

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met$_1$ is greater than would have been expected from the frequency of recombination in the *pro*$_1$–*met*$_1$ and *met*$_1$–*cit*$_1$ intervals. It is still possible that the gene order is *lys*$_3$, *pro*$_1$, *cit*$_1$, *met*$_1$ rather than *lys*$_3$, *pro*$_1$, *met*$_1$, *cit*$_1$. The unusual phenomena associated with this chromosome, which, with chromosome *X* and unlike the other 8–10 chromosomes, remained disomic after PFP treatment, will be discussed in a later paper.

SUMMARY

Forty-two mutants of the anther smut fungus *Ustilago violacea* were mapped by means of complementation tests, mitotic haploidization, and meiotic segregation. Spontaneous mitotic haploidization was very rare, but haploids were induced at a high frequency using *p*-fluorophenylalanine (PFP). Haploid segregants appeared as fast-growing, spherical colonies (papillae) which grew away from the diploid growth on PFP medium. Thirty-three markers, classified by complementation tests into 21 genes, were mapped by mitotic haploidization in 10–12 linkage groups. There were no discrepancies in the linkage data, and all the markers could be assigned unequivocally to linkage groups. Although about 250 diploids were analysed, there were no segregants in which mitotic crossing-over and mitotic haploidization appeared to have occurred simultaneously.

Thirteen of the 33 markers, in six or seven genes, were expressed infrequently (0–5 %) in the papillae produced on PFP medium. These markers, which behaved unusually and were designated missing-markers, were found to be on two chromosomes which tended to remain disomic on PFP medium. Thus 8–10 chromosomes haploidize readily on PFP medium, whereas two other chromosomes are resistant to the effects of PFP and remain disomic. Meiotic segregation was investigated in crosses of genetically marked haploid stocks and also in diploids, using the host plant. Some of the results enabled preliminary maps to be made of three linkage groups. The results from meiotic segregation were fully compatible with those from mitotic haploidization and the complementation tests.

The genetical evidence for a haploid chromosome number of at least 10–12 is in conflict with the observations of several cytologists that $n = 2$ in this species.

This work was carried out while one of us (A. W. D.) was the holder of a Postgraduate Studentship from the Ministry of Agriculture, Fisheries and Food.

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