Extracted from: Moore, D. & Novak Frazer, L. (2002). *Essential Fungal Genetics.* Springer-Verlag, New York.

Chapter 5: Recombination analysis

5.1 Linkage studies make maps

If the recombination frequency is a measure of distance between two genes it should be possible to carry out a systematic series of crosses that will give you the recombination frequencies for pair wise combinations of a collection of different genes. From these, you should be able to construct a map of the relative positions of the genes on the chromosome. This proves to be perfectly feasible but there are a few points we need to make before illustrating the operation.

The first point is that there is a limit to the amount of recombination that can be recognized in a single cross. That limit is set by the outcome of a cross between two genes that are known to be located on different chromosomes. We have already explained in Chapter 4 that a cross between mutant strain *a* and mutant strain *b* will produce a diploid with the genotype:

$$\frac{a}{+} \frac{+}{b}$$

When such a diploid goes through meiosis, it will give rise to progeny of four genotypes in equal frequency, so a sample of the progeny population will contain the following genotypes:

25% [*a*+] (parentals)
25% [+*b*] (parentals)
25% [*ab*] (recombinants)
25% [++] (recombinants)

Obviously, the recombinant progeny amount to 50% of the total, yet we know, because we set this feature at the outset, that these genes are on different chromosomes. A consequence of this is that if we cross two unknown genes together and get a close to 50% recombinant genotypes we can only describe them as showing 'random assortment' or 'random segregation'. The two genes might be on different chromosomes and genuinely 'unlinked', but on the other hand they might be on the same chromosome (that is 'linked') but so far apart that crossover occurs between them in all meioses. Yes, that's right; one crossover somewhere between two linked genes occurring in each meiosis will give you 50% recombination.

So, 50% recombination is the upper limit, but statistical variation comes into this. We can use the equation in section 4.8 to calculate the minimum number of progeny required to distinguish between, say, 45% recombinant progeny and 50% recombinant progeny with different degrees of certainty. For 95% probability you will need to analyze a total of 1563 progeny. If you want to be 99% certain, you will have to analyze at least 2700 progeny. What this means, is that in normal practice, where only a couple of hundred progeny might be routinely analyzed, recombination values in the 40% region cannot be distinguished from random segregation with any great certainty.

Despite this limitation, it is feasible to intercross a collection of mutants and arrange them into linkage groups, that is, subsets of mutant strains which, in mutant × mutant crosses, give a recombination percentage that is (in statistical terms) significantly less than 50%. The recombination percentages can then be sorted into linkage group maps. For example, consider the small collection of imaginary recombination values shown in Table 5.1. The first three results contribute to a reasonable map, with the genes in the order *x-y-z*, as shown in Fig. 5.1. This little map is quite consistent with the data we have for these genes and, although it is not perfect, it is acceptably additive (15 + 5 approximates satisfactorily to 18). Bear in mind that in practice recombination

Table 5.1. Some imaginary recombination values	
Genes in the cross	Recombination %
x × *z*	18
x × *y*	15
y × *z*	5
w × *z*	43
w × *y*	45
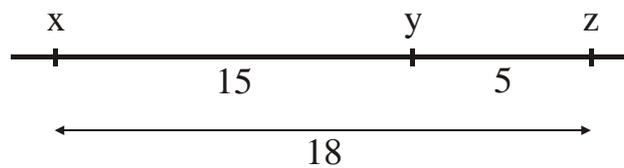
Fig. 5.1. A simple, and imaginary, three-point chromosome map.

frequency measurements such as these might have standard errors of about 10% of the value stated in the table, in which case this additivity equation would be more realistically stated as [(15 ± 1.5) + (5 ± 0.5) = 18 ± 1.8]. So we are reasonably satisfied with the map for these three genes.

That's all well and good, but what about gene *w*? With the data we have in the table, there are three possibilities: (i) unless a very large sample of progeny was analyzed, there's a possibility that 45% and 43% recombination are not significantly different from random segregation, so gene *w* may not even be linked to the other three; (ii) taking the 45% and 43% recombination frequencies at face value, gene *w* could be far to the right of gene *z*; but (iii) equally, gene *w* could be far to the left of gene *x*. We need more data to position gene *w*, but whatever its location it will be difficult to position reliably. We might consider making a *w* × *x* cross in the expectation that the result would indicate random segregation if gene *w* is to the right of *z*, but a recombination value of around 30% if gene *w* is to the left of gene *x*. However, remembering the variability expected in normal experiments, we would have to analyze a large number of progeny, probably from several hundred to a couple of thousand, if we want to obtain a believable result. But there is a better way.

Much more information is obtained from a cross if three rather than two genetic markers are followed. In three-point crosses, recombination frequencies are obtained in the usual way but they also allow the relative order of the genes on the chromosome to be determined, just by direct inspection of the data. To see why this is so we will start by using the basic knowledge we already have about recombination to predict the outcome of an imaginary three-point cross. We should then be able to recognize the underlying rules that will allow us to interpret the results of real-life crosses.

5.2 Multipoint crosses

For our imaginary cross we will use the imaginary genes *a*, *b* and *c*. We are going to assume that they are all linked together with 10% recombination between *a* and *b*, and 10% recombination between *b* and *c*. The map for this region is shown in Fig. 5.2a. We are planning to cross a strain carrying mutations in all three genes (a triple mutant strain) with the wild type, so the heterokaryon will be [*abc*] + [+++] and the parental diploid nucleus, just before the start of meiosis, will have the appearance shown in Fig. 5.2b.

The first task is to predict the full range of genotypes that meiosis can produce and which are expected to appear among the progeny in consequence. The simplest thing that can happen during meiosis is that the homologous chromosomes replicate and the daughter chromatids disjoin without recombining. When that happens, progeny of parental genotypes are produced, namely *abc* and +++.

But in some meiotic divisions (about 20% of them, but we will deal with the frequencies later) a recombination event will occur between genes *a* and *b* at the four-strand stage (Fig. 5.2c), giving rise to the recombinant genotypes +*bc* and *a*++.

Similarly, in other meiotic divisions (again, about 20%) a recombination event will occur between genes *b* and *c* at the four-strand stage (Fig. 5.2d). This event will produce the recombinant progeny genotypes *ab*+ and ++*c*.

Remember that recombination mapping like this assumes that recombination events occur at random. Now, if recombination between *a* and *b* is occurring at random, and recombination between *b* and *c* is also occurring at random, then there must be some meiotic divisions in which **both** of these recombination events occur at the four-strand stage (Fig. 5.2e). This situation will produce the recombinant progeny genotypes *a*+*c* and +*b*+. We have now predicted all the expected genotypes, and they have been brought together into Table 5.2.

We can use the information we have about recombination frequencies to calculate the expected frequency of each of these progeny types, starting with the double crossovers. We know that there is 10% recombination between *a* and *b*, and 10% recombination between *b* and *c*. Now, if recombination really does occur at random, then a recombination frequency is a probability statement. That is, 10% recombination is equivalent to saying that there is a 10% probability of a recombination event occurring between *a* and *b*, and, in this case of course, there is also a 10% probability of a recombination event occurring between *b* and *c*. Two truly random events occur independently of one another. That is, if event *x* is really random, then it must occur without reference to a separate event, *y*, and the probability of both events occurring together is the algebraic product of their separate probabilities. In our example, that works out to 10% of 10%, which is 1%.
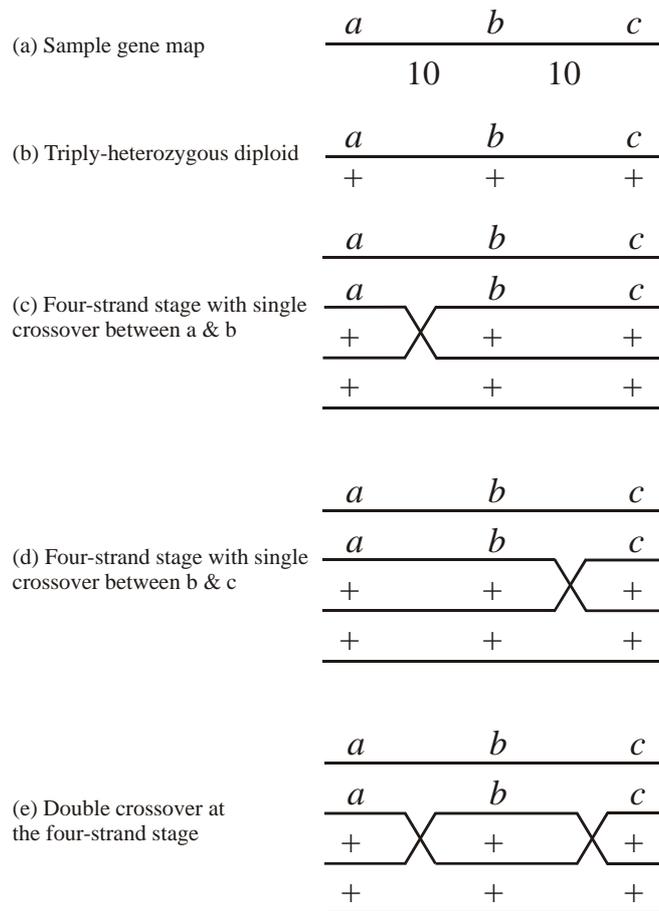
Fig. 5.2. Predicting the outcome of a three-point cross. (a) The disposition of the genes; (b) full genotype of the triply-heterozygous parental diploid nucleus, just before the start of meiosis; (c) a recombination event between genes *a* and *b* at the four-strand stage; (d) a recombination event between genes *b* and *c* at the four-strand stage; (e) both of these recombination events occurring at the four-strand stage.

Table 5.2. Summary, origins of genotypes, and predicted frequencies of progeny expected from the imaginary cross illustrated in Fig. 5.2

Genotype	Nature and/or origin	Frequency (%)
abc	parental, formed by chromatid disjunction only	40.5
+++	parental, formed by chromatid disjunction only	40.5
a++	single cross-over in interval I (between *a* and *b*)	4.5
+*bc*	single cross-over in interval I (between *a* and *b*)	4.5
ab+	single cross-over in interval II (between *b* and *c*)	4.5
++*c*	single cross-over in interval II (between *b* and *c*)	4.5
a+*c*	double cross-over	0.5
+*b*+	double cross-over	0.5

Be careful when you are calculating with percentages to remember that they are proportions, not whole numbers. It's probably safer to convert them to decimal fractions of one for calculation, so that 10% of 10% becomes $0.1 \times 0.1 = 0.01$. Whichever route of calculation you use, the end product is that 1% of the progeny will arise from double crossovers. Since crossovers are reciprocal, the two genotypes that result from double crossovers will each form half the total: there will be 0.5% *a*+*c*, and 0.5% +*b*+.

For the single crossover genotypes it's not as simple as assuming that their frequency is the same as the recombination frequency. The double crossovers provide the complication. Each double crossover progeny, by definition, represents a crossover in each interval. A recombination frequency of 10% in an interval means that the sum total of all crossovers, single and double, amounts to 10%. It follows, then, that the expected frequency of single crossovers in interval I (between *a* and *b*) is 10% (the recombination frequency) minus 1% (the

expected frequency of double crossovers) = 9%. Again, the crossovers are reciprocal, so the two genotypes that result from this crossover will each form half the total: there will be 4.5% +*bc* and 4.5% *a*++. To ease the calculation in this example we made the recombination values the same in the two intervals; that's not likely to be the case very often, of course, but right now the expected frequency of single crossovers in interval II (between *b* and *c*) is 10%, subtract 1% (the expected frequency of double crossovers is used again because they do, after all, have two crossovers), resulting in 4.5% *ab*+ and 4.5% ++*c*.

To work out the expected frequency of progeny with the parental genotypes, total up the recombinants we've just calculated and subtract from 100%. The complete set of expected progeny frequencies are listed in Table 5.2. This completes the predictions for the progeny from this cross. Now let's extract the general rules for analyzing three-point crosses.

5.3 Rules of the three-point crosses game

Progeny from a three-point cross always fall into eight genotypes, which are in four pair wise combinations of reciprocal genotypes. When the three genes in the cross are linked, the progeny types appear in a very characteristic pattern of frequencies.

- One pair of genotypes is always in a large majority. These are the parental genotypes. They reveal the exact genotypes of the strains used to make the cross and, consequently, the exact genotype of the triply-heterozygous diploid nucleus.
- One pair of genotypes is always in a small minority. These are the genotypes that arise from double recombinants and they are always the least frequent classes of progeny because they do require two recombination events.
- There are then two pairs of genotypes that arise from single recombinants. The numbers of progeny in these classes depends on the distances between the genes on the chromosome. A large interval between two genes will contain many crossovers and produce many singly recombinant progeny. A small interval will contain few crossovers and correspondingly few singly recombinant progeny. If the two intervals are about the same size then these classes will also contain about the same number of progeny, as in our theoretical example, above.

The progeny frequencies can, obviously, be used to calculate the recombination frequencies and map the genes. But there is an important piece of information that can be obtained from inspection of the data before the calculation starts; and that is the order of the genes. Three genes can be arranged in three different orders (e.g. *a-b-c*, *a-c-b*, or *b-a-c*). In most cases when you set out to perform the cross you will not know the order of the genes in advance; it's one of the pieces of information you need to get from the results of the cross. And you can infer the order by comparing the genotypes of the parentals with the genotypes of the double recombinants. This is because the parental genotypes show you the chromosomal arrangements that entered the meiosis, and the double recombinants reveal the chromosomal arrangements resulting from the original after two crossovers, one on either side of the middle gene. It is specifically the central marker in the set of three that the two recombination events re-arrange. So the trick is to compare the parental genotypes with the double recombinant genotypes; only one gene will have changed its arrangement relative to the others, and that gene is the one that's in the middle.

Look back at our theoretical example; *abc* and +++ occur in the greatest frequency and are the parentals. The least frequent genotypes are *a*+*c* and +*b*+; these are the double recombinant genotypes and comparing them with the parentals (compare *a*+*c* & *abc* and +*b*+ & +++) shows that gene *b* is in the middle of the three. But that was easy; we knew that from the start! Let's try a genuine sample three-point cross.

5.4 A three-point cross in *Coprinus*

This experiment used three nutritional mutants, *met*-1, a methionine auxotroph, *ade*-2, an adenine-requiring mutant and *pdx*, an auxotroph that cannot synthesize pyridoxine (vitamin B$_6$; used as a coenzyme for many enzymes, especially transaminases and decarboxylases). The cross was set up between the double mutant strain *pdx*, *ade*-2 and the *met*-1 isolate. The basidiospore viability was a satisfactory 87%, and a total of 411 progeny were analyzed, yielding the progeny genotype frequencies shown in Table 5.3. The numbers observed in the progeny classes corresponding to the genotypes *pdx ade*-2 + and + + *met*-1 confirm the (given) parental genotypes. It is equally evident, in terms of the numbers observed, that + *ade*-2 + and *pdx* + *met*-1 are the double recombinants. Comparing these genotypes shows that *pdx* is the central gene of the trio. Compare parental + + *met*-1 with double recombinant *pdx* + *met*-1; or compare parental *pdx ade*-2 + with double recombinant + *ade*-2 +; in each case *pdx* has changed its relationship with the other two genes; it has been recombined. As it is the central gene that recombines in double recombinants, it follows that the gene map must be *ade*-2 -- *pdx* -- *met*-1.

Table 5.3. Progeny from a three-point cross in *Coprinus cinereus*	
Progeny genotype	Number
pdx, *ade*-2, +	155
+, +, *met*-1	170
pdx, +, +	36
+, *ade*-2, *met*-1	30
pdx, *ade*-2, *met*-1	7
+, +, +	10

To work out the recombination frequencies, it is convenient to restructure the table of results to deal with the progeny in two pair wise combinations, *ade*-2 to *pdx*, and *pdx* to *met*-1. This gives the following progeny distributions:

pdx	ade-2	155 + 7
+	+	170 + 10
pdx	+	36 + 1
+	ade-2	30 + 2

Which corresponds to 69 recombinants in 411 progeny = 16.8% recombination. And,

pdx	+	155 + 36
+	met-1	170 + 30
pdx	met-1	7 + 1
+	+	10 + 2

Corresponding to 20 recombinants in 411 progeny, or 4.9% recombination. So the final map can be drawn like this: *ade*-2 -- 16.8 -- *pdx* -- 4.9 -- *met*-1.

There is one further piece of information we can extract from these data. When we first calculated the expected frequency of double recombinant progeny in section 5.2, we did it by multiplying together the two recombination frequencies with the assumption that recombination events in one interval were independent of recombination events in the other interval. We have here a means of testing that assumption. We know that there were 3 double recombinant progeny (= 0.73%), and we can calculate that we expect 16.8% of 4.9% double recombinants (= 0.82%). Clearly, the two do not match. We observe fewer double recombinant progeny than the assumption of randomness in recombination leads us to expect. This is quite commonly observed. What it means if that crossing-over within one interval is not independent of crossing-over within the immediately adjacent interval. Instead, there is some sort of interference between the two crossover events such that the occurrence of one crossover makes a second crossover in the immediate vicinity less likely to occur. This is called chiasma interference because it reduces the occurrence of chiasmata (the fancy name for crossovers). What causes it is not known, but the extent of it is measured with a value called the coefficient of coincidence, calculated by comparing the expected frequency of double crossing-over with the observed frequency:

$$\frac{\text{observed frequency of double crossing - over}}{\text{expected frequency of double crossing - over}}$$

If crossing-over in one interval is fully independent of crossing-over in the other, you will observe as many double recombinants as you expect and this coefficient will calculate to the value 1. However, if there is interference between crossovers, such that crossing-over in one interval reduces the likelihood of crossing-over in the other, the coefficient will be less than 1. This is what we have found in the *ade*-2- *pdx* - *met*-1 region of the *Coprinus* chromosome. The coefficient of coincidence here is calculated with this equation.

$$\frac{0.0073}{0.0082} = 0.89$$

This corresponds to a mild but positive interference. Calculating the coefficient of coincidence is therefore a means of detecting interference between crossovers. It is usually mildly positive (with coefficients of coincidence in the 0.6 to 0.9 range) although if the region of chromosome you are working with is located near a recombination hot-spot you may observe more double recombinants than expected which would give a coefficient of coincidence greater than one. This is called negative interference and has been observed, but only occasionally.

The mapping process we have just described, using two- and three-point crosses, is applicable to all eukaryotes. The only difference is a practical one arising from the fact that most animals and plants are diploid in their free-living phase. In haploid fungi, progeny genotypes are immediately evident in the phenotypes of the haploid progeny. In diploid organisms though, testcrosses, in which one of the parents carries the recessive alleles of all of the genes under investigation, must be used to ensure that phenotypes of the diploid progeny directly represent genotype distributions of the gametes arising from the triply heterozygous parent.

There is one aspect of chromosome mapping that is almost totally limited to fungi, in the sense that only fungi offer a means to perform the analysis as part of their normal life style. This is the ability to map the position of the centromere within its linkage group using progeny segregation data.

5.5 Mapping centromeres using gene segregations in tetrads and eight-spored asci (octads): single gene segregations

A unique feature of many fungi is that it is possible to isolate and analyze all four products of a single meiosis. A few ascomycetes are even more unique because their ascus is so narrow that all division spindles are constrained to its long axis. As a result, in these few species the meiotic products are arranged in a linear order that strictly depends on the mechanics of meiosis (Fig. 5.3). This happens in species of *Sordaria*, *Ascobolus* and a few species of *Neurospora*.

Frequencies of these patterns depend on the frequency of recombination between the gene and its centromere. So, by recording the number of second division segregating asci, the distance between the gene and its centromere can be calculated. Unfortunately, the convention for calculating recombination frequency (total recombinant progeny as a percentage of total progeny) was established before these ascus segregations were identified. Since half the spores in a second division segregation ascus are non-recombinant, the recombination percentage is equal to the second division segregation percentage divided by two.

As an aside, note that it is not just ascus segregations to which these statements apply, but to meiosis in all eukaryotes. Recombination occurs at the four-strand stage of meiosis, but one crossover only ever involves two of the four chromatids. The other two chromatids remain non-recombinant, and therefore contribute to the divisor in the recombination frequency equation. So as a general rule the percentage of meioses in which a crossover occurs equals twice the recombination frequency. A recombination frequency of 20% means that 40% of meioses have a crossover in that interval; and a recombination frequency of 45% corresponds to 90% of meioses having a crossover between those genes. Not convinced? Well, let's work it out this way: say you have 100 meiotic divisions and 10 of them (= 10%) have a crossover between two particular genes; what is the recombination frequency? 100 meioses produce 400 chromatids, each one of which will enter a spore and become a progeny mycelium. In the ten recombinant meioses the crossover will take place at the four-strand stage, but will involve only two strands in each meiosis, so total recombinant progeny will be $10 \times 2 = 20$. Consequently, the recombination frequency calculates to:

$$\frac{20}{400} \times 100\% = 5\%$$

But we started out by saying that 10% of the meioses had a cross-over between those two genes ... it's all a matter of arithmetic.

How ascus segregations are used for mapping is illustrated in the following example. Ascospore colour in *Sordaria* is controlled by a pair of alleles at one gene locus, the wild type allele, +, makes spores black and the mutant allele, *w*, results in a white spore. Suppose that a cross was made between a white and a black isolate and the segregation of spore colour in 274 asci formed by that cross was as shown in Table 5.4.

You have to be careful reading data presented like this. Each vertical column represents 'an ascus type' and the cells within each column represent the spores in that ascus type. Since an ascus is a hyphal branch, it has a recognizable bottom (where it is joined to the parental hypha) and a recognizable top (the ascus apex, where the growing tip of the branch used to be). We have numbered each ascospore in Table 5.4, so that ascospore 1 is
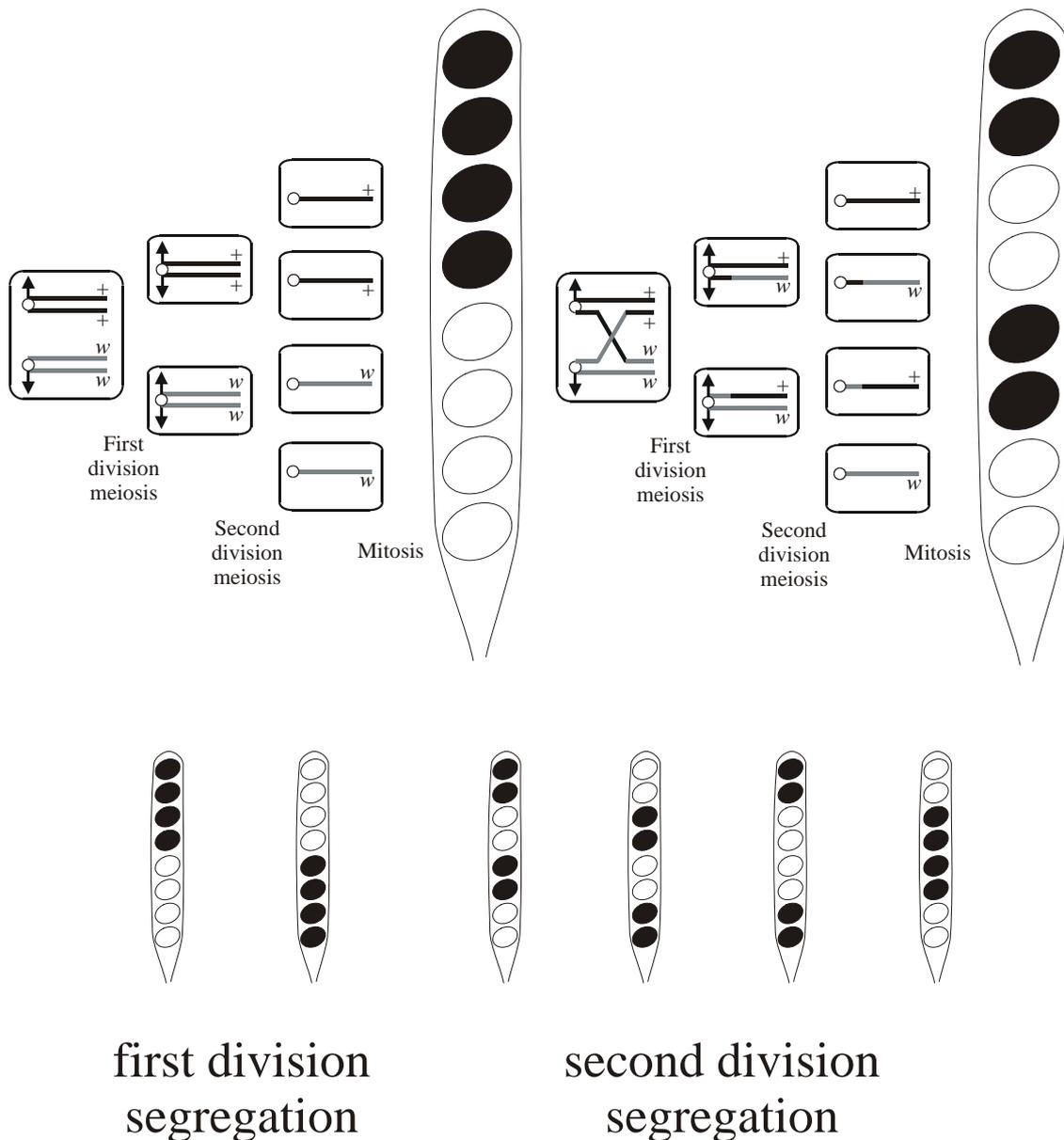
Fig. 5.3. Ascus segregation patterns of ascomycetes like *Sordaria*, *Ascobolus* and a few species of *Neurospora*, illustrated with the segregation of a spore colour phenotype (+ = wild type black, *w* = white). All division spindles are constrained to the long axis of the ascus. As a result, in these few species the meiotic products come to be arranged in a linear order that strictly depends on the mechanics of meiosis and whether or not a crossover occurs between the gene and its centromere (top panel). Because the ascus has a recognizable top and bottom, and nuclei can rotate before dividing, there are two first division and four second division segregation patterns (bottom panel).

at the top and ascospore 8 at the bottom. The extreme right hand column of Table 5.4 is telling you that in the sample of asci examined, there were 5 that had two white ascospores at the top, followed by two black, then two white, and finally two black at the bottom. That's a second division segregation pattern. The next column in from the right shows that there were 105 asci that had four black ascospores in the top half of the ascus and four white in the bottom half. This is a first division segregation pattern. Carry on interpreting the columns in the table like that and you will see that both first division segregation patterns are represented (both shaded in the table), and all four possible second division segregation patterns.

Table 5.4. Progeny octads obtained from a cross in *Sordaria* between the wild type allele, **+**, which makes the spores black and the mutant allele, *w*, which results in white spores.

|	Ascus type (first or second division)					
	2nd	2nd	1st	2nd	1st	2nd
ascospore 1	+	w	w	+	+	w
ascospore 2	+	w	w	+	+	w
ascospore 3	w	+	w	w	+	+
ascospore 4	w	+	w	w	+	+
ascospore 5	+	+	+	w	w	w
ascospore 6	+	+	+	w	w	w
ascospore 7	w	w	+	+	w	+
ascospore 8	w	w	+	+	w	+
Totals observed	9	16	129	10	105	5

The data we use for calculation are the totals in the final (bottom) row. These establish that first division segregation asci total 129 + 105 = 234, and second division segregation asci total 9 + 16 + 10 + 5 = 40. Consequently:

$$\% \text{ second division segregation } = \frac{40}{274} \times 100 = 14.6\%$$

Now, remember that in each second division ascus only half the spores derive from chromatids that have undergone crossing over, so the recombination percentage is half of this second division segregation frequency; therefore, the spore colour locus shows 7.3% recombination with its centromere.

5.6 Mapping using multiple gene segregations in tetrads and octads

It is also possible to use ascus segregations to study linkage between chromosomal genes. We've called the mutant alleles *a* and *b* in the following examples; so consider two genes with mutant and wild type alleles *a*/+ and *b*/+. If we make a cross and then analyze spores from individual asci after recording the position of each spore in its ascus, then the possible segregation patterns from the cross *ab* × ++ are as shown in Fig. 5.4. There is

Parental ditype   Non-parental ditype   Tetratypes
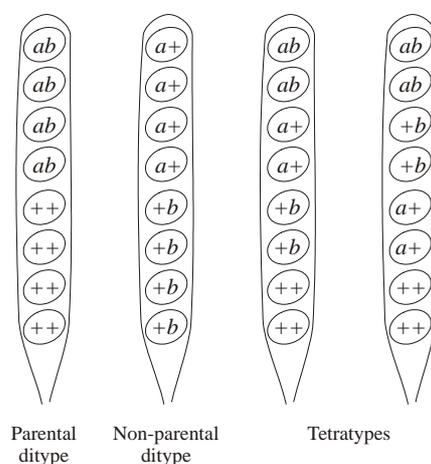
Fig. 5.4. Possible segregation patterns in octads from the cross *ab* × ++.

the parental ditype (PD), which has just 2 types of spore with the parental genotypes; the non-parental ditype (NPD), again with two types of spore, but this time all spores have recombined (or non-parental) genotypes; and the figure shows two forms of tetratype (T), which contain four types of spore, comprising all of the possible genotypes. The relative frequencies of these ascus types depend on whether the genes are linked.

If the genes are unlinked, then: (a) chromosomes assort independently and PD as well as NPD arise with equal frequency; and (b) tetratypes arise when one gene recombines with its centromere. The two sorts shown in Fig. 5.4 show the two alternatives of either gene a or gene b recombining with its centromere and segregating in the second division of meiosis.

If the genes are linked, then (a) PD arise by chromosome segregation and are common; (b) NPD only arise through a four-strand double crossover and are rare; and (c) tetratypes arise from a single crossover between the genes.

The formula for calculating recombination percentages from this sort of octad analysis is therefore:

$$\% R = \frac{\frac{1}{2}T + NPD}{Total\ Octads} \times 100$$

Since only two chromatids in a tetratype are recombinant the number of tetratypes must be divided by two, on the other hand, all spores in NPD asci arise from recombinant chromatids so they all count as recombinant progeny in this equation.

Tetrad analysis is a very sensitive means of detecting linkage. This is because a PD: NPD ratio of 1: 1 is absolutely diagnostic of no linkage. If this ratio is significantly different from 1: 1 then linkage is indicated.

For an example of this sort of analysis we will turn to *Neurospora crassa*, and an experiment in which a strain unable to synthesize riboflavin (symbolized *ribo*; riboflavin is vitamin B_2 and contributes to flavin nucleotide coenzymes) was crossed with a strain unable to synthesize the amino acid tryptophan (*tryp*). The asci that were scored are shown in Table 5.5. Again, each ascus type is represented by the eight spore genotypes in each vertical column, but in this case, no distinction has been made between the top and bottom of the ascus (to reduce the number of columns needed to display the results), though we have numbered each column so that we can refer to particular ascus types when required.

We can use these data to construct a chromosome map showing the positions of *ribo*, *tryp* and their centromere, and we can also extract a bit more information from these data. Taking it one step at a time, first consider the relationship between *ribo* and *tryp*. Ascus types 1, 2, 3 and 4 are all parental ditype, and all of the rest are tetratypes. There are no non-parental ditypes, so clearly the two genes are linked together and presumably they are too close together for any NPD asci to appear in a sample of this size. Converting these statements to numbers we have: PD = 129 + 1 + 2 + 1 = 133; NPD = 0; T = 15 + 13 + 17 + 17 + 2 + 1 = 65, total asci scored = 198; so *ribo* and *tryp* are linked and are 16.4 units apart. But these are octads, so we can determine the position of the centromere by re-interpreting the data in terms of segregation at first or second division of meiosis.

The second step, then, is to examine the relationship between *ribo* and the centromere. We are only interested in second division segregation asci, and looking back at Table 5.5, ascus types 2, 3, 4, 9 and 10 have *ribo* segregating in the second division. In numbers this = (1 + 2 + 1 + 2 + 1) = 7, and 7/198 = 3.5%. Therefore the distance from *ribo* to the centromere is half this, which is 1.8% recombination.

Applying the same arguments to the relationship between *tryp* and the centromere: second division segregation asci total (1 + 2 + 1 + 15 + 12 + 17 + 17 + 1) = 67, and 67/198 = 33.8%, so it follows that *tryp* shows 16.9% recombination with the centromere. Putting all this together we can draw the map: centromere -- 1.8 -- *ribo* -- 16.4 -- *tryp*, which is a very nice little map, until somebody asks: 'since when did 1.8 + 16.4 add up to 16.9?'

Indeed, this map illustrates an extremely common feature, that recombination in longer intervals tends to be underestimated. But these are whole asci, and their spore arrangements record all of the events in meiosis. Because they record all events, octads allow us to carry out a complete audit of recombination events. Nothing that follows this should be taken to imply that the approach and analysis above is wrong. The asci were categorized correctly and calculations done properly. In experiments using random spores the analysis could not be taken any further because there are no further data. But these ascus segregations reveal instances of double recombination that would otherwise go undetected.

For example, 67 asci were categorized as second division segregation between *tryp* and the centromere. But refer to Table 5.5 and you will see that asci numbers 9 and 10 contain some surprises. Ascus 10, for example, has a distribution of spore genotypes that can only be explained as resulting from a three-strand double crossover. This has already been included among the 67 single crossover second division segregation asci, but it represents two cross-over, so we must add one for the second crossover; and total recombination events between *tryp* and the centromere so far total 67 + 1 = 68.

Table 5.5. Progeny octads obtained from a cross between a strain of *Neurospora crassa* unable to synthesize riboflavin (*ribo*) and a strain unable to synthesize the amino acid tryptophan (*tryp*)

Ascus type (reference number)									
1	2	3	4	5	6	7	8	9	10
ribo +	*+ tryp*	*ribo +*	*ribo +*	*ribo tryp*	*ribo tryp*	*ribo +*	*ribo +*	*ribo +*	*ribo tryp*
ribo +	*+ tryp*	*ribo +*	*ribo +*	*ribo tryp*	*ribo tryp*	*ribo +*	*ribo +*	*ribo +*	*ribo tryp*
ribo +	*ribo +*	*+ tryp*	*+ tryp*	*ribo +*	*ribo +*	*ribo tryp*	*ribo tryp*	*+ +*	*+ +*
ribo +	*ribo +*	*+ tryp*	*+ tryp*	*ribo +*	*ribo +*	*ribo tryp*	*ribo tryp*	*+ +*	*+ +*
+ tryp	*ribo +*	*ribo +*	*+ tryp*	*+ +*	*+ tryp*	*+ +*	*+ tryp*	*ribo tryp*	*ribo +*
+ tryp	*ribo +*	*ribo +*	*+ tryp*	*+ +*	*+ tryp*	*+ +*	*+ tryp*	*ribo tryp*	*ribo +*
+ tryp	*+ tryp*	*+ tryp*	*ribo +*	*+ tryp*	*+ +*	*+ tryp*	*+ +*	*+ tryp*	*+ tryp*
+ tryp	*+ tryp*	*+ tryp*	*ribo +*	*+ tryp*	*+ +*	*+ tryp*	*+ +*	*+ tryp*	*+ tryp*
129	1	2	1	15	13	17	17	2	1

The two octads in category 9 have the first division segregation pattern for *tryp* and, for that reason, have not been included in the calculation of recombination between *tryp* and its centromere at all yet. These asci, however, do show second division segregation for *ribo*, and have been included in that calculation. But think about that fact; *ribo* is closer to the centromere than *tryp* and we know that the second division segregation for *ribo* means that these asci have a crossover between *ribo* and the centromere. The only way that the outer gene can segregate in the first division when the inner segregates in the second division is if a second crossover takes place between the two genes. So these two asci each arise from two-strand double crossovers between *tryp* and its centromere. A total of four crossovers that have not been included yet, and must be added in to bring the overall total count of recombination events between *tryp* and the centromere to 68 + 4 = 72. This allows corrections of the recombination values as follows.

Between *tryp* and centromere, recombination percentage $= \dfrac{\frac{1}{2}72}{198} \times 100$

$= 18.182\%$.

Between *ribo* and *tryp*, recombination percentage $= \dfrac{\frac{1}{2}65}{198} \times 100$

$= 16.414\%$.

Between *ribo* and centromere, recombination percentage $= \dfrac{\frac{1}{2}7}{198} \times 100$

$= 1.768\%$.

Which gives us perfect additivity: 1.768 + 16.414 = 18.182. The message of this, rather heavy-handed, analysis is that the commonly encountered underestimation of recombination percentage over longer intervals is due to the occurrence of double crossovers. The second crossover reverses the genotype change caused by the first with the result that the fact that the progeny are recombinant is no longer apparent.

Analysis of spore segregations in asci has revealed a great deal about eukaryotic genetic mechanisms.

An immediately relevant example is that octad analysis enables the distribution of crossovers to be established; the spore patterns arising from two-strand double, the two forms of three-strand double, and four-strand double recombinants can all be distinguished and counted (Fig. 5.5). All of these are found to occur and they occur at equal frequency. This means that there is no limitation in the choice of chromatids by successive crossovers, that is, there is no chromatid interference.

Furthermore, although the six patterns shown in Fig. 5.3 are normally expected from crosses between alleles of the same gene (e.g. black × white spore character), in rare asci aberrant segregations including 5 black: 3 white are observed. Explanation of how such octads can arise reveal some details of the mechanism of recombination, which we will discuss in Chapter 6.
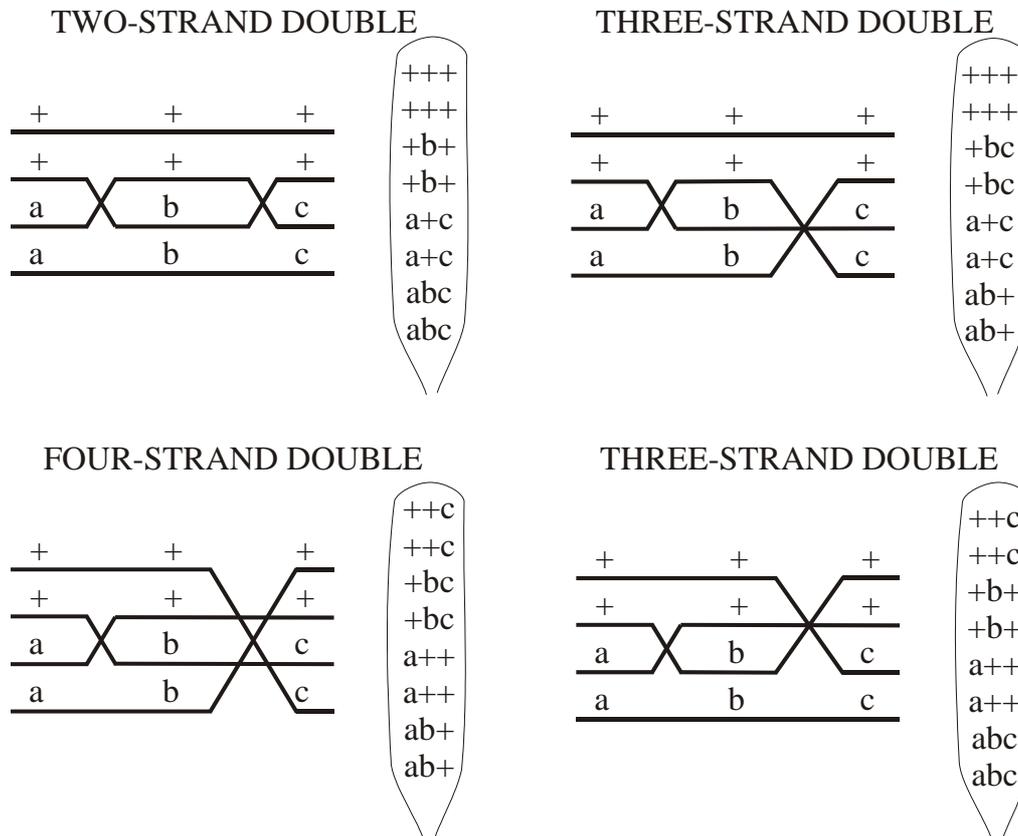
Fig. 5.5. Line diagrams of the four different types of double crossover events, shown here as occurring at the four-strand stage (prophase of meiosis) in a region of the chromosome heterozygous for mutant genes *a*, *b* and *c*. The lists on the right show the genotypes of ascospores, as they would appear in ascus octads following these recombination events. Each pattern is distinct, so all of the double crossover events can be identified and their frequencies of occurrence determined.

5.7 Unordered tetrads

Isolating the spores from the ascus one by one in order, noting their original positions, and then testing their colonies after germination for whatever phenotypic characters are segregating in the cross quite readily establish ascus spore segregation patterns. The initial ascospore isolation needs to be done with the aid of a microscope, and may require a micromanipulator, so the technique can be tedious, but it is not especially difficult. Since the order of the four chromatids generated by the meiotic division is maintained by the shape and size of the ascus (even after the post-meiotic mitosis), these are called ordered octads. As we have noted, asci narrow enough to maintain the inherent order of meiosis like this are produced by very few species. Many, many other species produce 'fatter' asci in which the spores are jumbled up. Then think of all those basidiomycetes in which meiotic products are packaged into basidiospores that are neatly arranged in sets of four on top of the basidia; there is no clue left here of any inherent meiotic order. These are called unordered tetrads because the meiotic order has been lost. Yet in each of these cases the products of individual meioses are kept together and all that has been said above about segregation in meiosis still, basically, applies.

The analysis of the basidiospore tetrads of *Coprinus*, for example, falls into four consecutive procedures: (i) tetrads must be isolated from fruit body tissues and the spores separated from one another; (ii) the spores must be germinated; (iii) the sporelings are grown up into colonies; and (iv) the colonies are tested. Basidiospore tetrads can be picked off from fresh fragments of the gills with a glass needle, relying on

electrostatic attraction for their physical removal. The needle is a thin filament of ordinary white soda glass with a small ball (about 40 μm diameter) on the end. It is necessary to use a micromanipulator because of the small size of the tetrads (approximately 10 μm square) and their high numerical density on the gill surface approximately 3,000 mm^{-2}).

Immediately after removal from the gill, the tetrad is placed onto an agar surface and the four spores separated from one another with the micromanipulator needle. All of these steps need to be done with the aid of a binocular dissecting microscope. There are two main difficulties inherent in this method of picking-off directly from the gill surface: the high numerical density of tetrads makes it very difficult to be sure that no extraneous spores have been picked-off until the tetrads are parked on the agar (by which time it is too late!); also, it is impossible to distinguish unripe from ripe tetrads so that many abortive attempts to pick up tetrads might be made before one is successfully removed.

In addition to these difficulties there are problems arising from poor spore germination and relatively high losses to contamination. It is difficult to isolate large numbers of tetrads because of such technical difficulties. Another point is that because the members of a tetrad do not represent independent events, the analysis of 100 tetrads (quite a formidable undertaking in itself) is not equivalent to the analysis of 400 random spores. In fact, on average, one tetrad contributes about as much data as two random spores. Thus, in comparison with random spore analyses, tetrad analysis suffers from the effects of small sample sizes, so when mapping from tetrad analysis data, the recombination values obtained are usually only approximate. However, it is still the only way of mapping centromeres from segregation data.

The method of analysis used to extract the required data from unordered tetrad segregations is slightly different from that used with ordered tetrads. It is an indirect method in that it is necessary to have other genes segregating in the same tetrad as the gene under test. There is a requirement for one other unlinked gene that is known to be close to its centromere so that it nearly always segregates at the first division (known as a centromere marker). Alternatively, at least two other genes are required in the cross, neither of which need be known centromere markers, but at least one of which must be independent of the gene whose centromere distance is to be determined.

The mathematical theory underlying the approach depends on the relative frequencies of the three different types of tetrad obtainable from a cross heterozygous at two loci, discussed at the beginning of section 5.6 above: the parental ditype (PD), the non-parental ditype (NPD), and the tetratype (T). The order of the spores in the tetrad is irrelevant to identifying these three categories. If there are four spore genotypes in the set, it must be a tetratype; if there are only two spore genotypes, it must be one of the ditypes, and comparing the spore genotypes with the parent genotypes will show you which ditype. However, different frequencies of these tetrad types can occur depending on the relative positions of the genes to each other and to their centromeres. As stated above, if the two genes are linked then the proportion of tetratypes is dependent on the frequency of crossing over in the interval between them, and with linked genes non-parental ditypes arise only from four-strand double exchanges between them.

If the two genes are on different chromosomes, then the tetratype frequency is a reflection of the rate of crossing over between the genes and their respective centromeres. If both genes are very close to their centromeres, tetratypes will be rare. Tetratype frequencies become greater as genes with larger centromere distances are used. Indeed, the rules are that tetratype tetrads arise in all cases where the genes individually show second division segregation and in half of the cases where both segregate at the second division simultaneously. If we can convert that into a mathematical statement we could use it for analyzing crosses.

Let's say we have two unlinked genes, *a* and *b*, that have second division segregation frequencies **a** and **b** respectively. It's fairly clear from the diagrams in Fig. 5.3 that a tetratype tetrad will be produced by a crossover between *a* and its centromere when *b* remains unrecombined. Now, the frequency of *b* remaining unrecombined is given by (1-**b**) and a proportion a of those will have a crossover between *a* and its centromere, so the frequency of tetratypes due to recombination of *a* alone = **a**(1-**b**). By a similar line of argument, the frequency of tetratypes due to recombination of *b* alone = **b**(1-**a**). The proportion of meioses in which both genes recombine with their centromere is given by multiplying their second division segregation frequencies together = **ab**. But because of the independent segregation of daughter centromeres in the second meiotic division, these meioses give rise to a ratio of 1PD: 2T: 1NPD tetrads. That is, the frequency of tetratypes arising when both genes segregate at the second division simultaneously = **ab**/2.

The overall frequency of tetratypes is the sum of these three cases = **a**(1-**b**) + **b**(1-**a**) + **ab**/2, which resolves to the expression:

$$\text{tetratype frequency } = \boldsymbol{a} + \boldsymbol{b} - \frac{3\boldsymbol{ab}}{2}$$

If you have previous knowledge of either **a** or **b**, it is possible to determine the other directly using this equation. Consequently, if you have one reliable centromere marker, you can use it in this way to establish the centromere distance of any other unlinked gene.

But all is not lost if both **a** and **b** are unknown. By introducing a third independent gene into the cross the required second division segregation frequencies can be calculated without prior knowledge of any of them. With a third pair of alleles, and considering the genes two at a time, three equations of the form shown above are obtained, which then can be resolved into three further equations, each of which gives one of the second division segregation frequencies.

To illustrate, we will add gene *c* (with a second division segregation frequency **c**) to the *a* and *b* loci just considered. With *c* in the cross, there are three pair-wise combinations, so we can write three equations for the tetratype frequency (which we will call T_{ab}, T_{ac} and T_{bc}):

$$T_{ab} = \boldsymbol{a} + \boldsymbol{b} - \frac{3\boldsymbol{ab}}{2}, T_{ac} = \boldsymbol{a} + \boldsymbol{c} - \frac{3\boldsymbol{ac}}{2}, T_{bc} = \boldsymbol{b} + \boldsymbol{c} - \frac{3\boldsymbol{bc}}{2}$$

Solutions of these three simultaneous equations for **a**, **b** and **c** are as follows:

$$\boldsymbol{a} = \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - 6T_{ab} - 6T_{ac} + 9T_{ab}T_{ac}}{4 - 6T_{bc}}}\right]$$

$$\boldsymbol{b} = \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - 6T_{ab} - 6T_{bc} + 9T_{ab}T_{bc}}{4 - 6T_{ac}}}\right]$$

$$\boldsymbol{c} = \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - 6T_{bc} - 6T_{ac} + 9T_{bc}T_{ac}}{4 - 6T_{ab}}}\right]$$

These equations look worse than they really are; the arithmetic is essentially straightforward and all that's required for the analysis are the three tetratype frequencies. There are two solutions to each of these equations. Negative ones are obviously unreal, and any positive value greater than 0.67 is unlikely because second division values greater than 67% are extremely rare.

For our worked example we turn back to *Coprinus cinereus*, and crosses between a wild type and an auxotroph unable to synthesize nicotinic acid, called *nic*-4, in which the mating type of all progeny was scored so that the *A* and *B* mating type factors made up the total of three genes. A total of 336 tetrads were analyzed. They were scored in a number of batches, so before doing the overall analysis it was necessary to verify the homogeneity of the data using contingency tables of the tetrad types between each gene pair observed in each batch. The χ^2 values obtained corresponded in each case to a probability of about 20% that the data were satisfactorily homogeneous. Correspondingly, the data were bulked into Table 5.6.

Table 5.6. A bulked progeny sample of 336 unordered tetrads of *Coprinus cinereus* scored for the auxotroph *nic*-4, and the *A* and *B* mating type factors

Gene pair	Tetrad types			
	PD	NPD	T	% Tetratypes
A/B	100	99	137	40.8
A/nic-4	92	99	145	43.2
nic-4/*B*	136	132	68	20.2

Testing the three PD: NPD ratios for deviation from 1:1 gave χ^2 values of 0.005 (*A/B*), 0.26 (*A/nic*-4), and 0.06 (*nic*-4/*B*). With one degree of freedom, these χ^2 values correspond to very high probabilities that deviations from 1:1 were due to chance variation. Consequently, the PD: NPD ratios provide good evidence that the three genes are unlinked, and we can continue with the analysis.
The second division segregation frequency (SDSF) of mating type factor *A* is given by this equation:

$$= \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - (6 \times 0.408) - (6 \times 0.432) + (9 \times 0.408 \times 0.432)}{4 - (6 \times 0.202)}}\right]$$

$$= \frac{2}{3}\left[1 \pm \sqrt{\frac{0.546}{2.788}}\right] = \frac{2}{3}\left[1 \pm \sqrt{0.196}\right] = \frac{2}{3}\left[1 \pm 0.443\right]$$

The two solutions of this equation are 0.962 or 0.372. The former is unreal (96% second division segregation?) which means that the mating type factor *A* shows 37.2% second division segregation (equivalent to a recombination frequency of 18.6%) with its centromere. Continuing to the other two genes in the cross, we have:

$$\text{SDSF of mating factor } B = \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - (6 \times 0.408) - (6 \times 0.202) + (9 \times 0.408 \times 0.202)}{4 - (6 \times 0.432)}}\right]$$

$$= \frac{2}{3}\left[1 \pm \sqrt{\frac{1.082}{1.408}}\right] = \frac{2}{3}\left[1 \pm \sqrt{0.768}\right] = \frac{2}{3}\left[1 \pm 0.876\right] = 1.25 \text{ or } 0.083.$$

and

$$\text{SDSF of } nic\text{-}4 = \frac{2}{3}\left[1 \pm \sqrt{\frac{4 - (6 \times 0.202) - (6 \times 0.432) + (9 \times 0.202 \times 0.432)}{4 - (6 \times 0.408)}}\right]$$

$$= \frac{2}{3}\left[1 \pm \sqrt{\frac{0.981}{1.552}}\right] = \frac{2}{3}\left[1 \pm \sqrt{0.632}\right] = \frac{2}{3}\left[1 \pm 0.795\right] = 1.20 \text{ or } 0.137.$$

Obviously, second division segregation frequencies in excess of 100% are unreal, so we conclude that mating type factor *B* shows 8.3% second division segregation (4.2% recombination) with its centromere, while the equivalent figures for *nic*-4 are 13.7% SDSF and 6.9% recombination.

This analysis reveals the very useful fact that mating type factor *B* in *Coprinus cinereus* is sufficiently close to its centromere for multiple recombination events to be rare. Since all crosses require heterozygosity in the mating type factors, and it is relatively simple to carry out mating type testing on the progeny, the B factor is a convenient centromere marker, which is 'built-in' to all crosses in *C. cinereus*.

5.8 Linkage analysis to linkage map
With a combination of random spore and tetrad analyses a good picture of the chromosomal locations of a wide range of functional genes can be established. Example chromosome maps for a few fungi can be found in several of the references we quote at the end of this chapter. The resolution and detail of maps constructed using genetic segregations is limited by the ability to analyze sufficient progeny to detect rare crossovers. The most detailed fungal segregation map is that of *Saccharomyces cerevisiae*, which shows the locations of approximately 1200 genetic markers. This is an average of about one for each 10 kbp of DNA. Some discrepancies became evident when the complete sequence of chromosome III of *S. cerevisiae* became available, but the discrepancies are very few in number, so for the most part segregation analysis gives an accurate view of gene arrangements.

Linkage analysis in *S. cerevisiae* is very efficient and it is unlikely that the resolution of the linkage map achieved in this yeast will be equaled in other fungi. Indeed in many cases the level of detail attained in yeast cannot even be approached in other fungi. The inaccuracies noted in the yeast linkage map when compared with the DNA sequence suggests that linkage maps cannot be expected to provide a detailed physical map of the DNA. Rather, the linkage map is a representation of the behavior of genes and chromosomes as they progress through the meiotic division cycle. This has enormous practical significance for our understanding of natural populations, prediction of progeny populations, and development of altered genotypes using classic genetic approaches.

The discussion so far has concentrated on functional genes, but much the same analyses using genetic segregations can be employed to construct gene maps showing the chromosomal locations of DNA markers such as restriction fragment length polymorphisms. However, such markers also lend themselves to alternative mapping techniques that depend on direct analysis of nucleic acid molecules, and we will discuss this in section 8.2.

5.9 Tetrad segregations leading to secondary homothallism

Some organisms use the mechanics of meiosis to control their sexuality. By producing fewer spores than there are nuclei to accommodate, nuclear migration after meiosis can make the spores homothallic. *Neurospora tetrasperma* is an example. Unlike its relative *N. crassa*, *N. tetrasperma* is homothallic, which means that the mycelium that grows from a single ascospore is self-fertile and is able to produces mature perithecia. The species shows secondary homothallism; this differs from the primary homothallism of *Aspergillus nidulans* and *Sordaria macrospora* because *N. tetrasperma* has a mating type locus that exists in two forms, A and a, and ascospores contain two nuclei, there being one of each mating type. The mating type locus is so close to its centromere that recombination between the two is extremely rare. Consequently, the mating type locus almost always segregates at the first division of meiosis. During the second meiotic division the division spindles are normally parallel, or at least overlap, and the subsequent mitotic division spindles are more or less at right angles to the long axis of the ascus. Ascospore walls surround one nucleus of each type. Thus, on germination, a single ascospore gives rise to a heterokaryotic mycelium that is heterozygous for mating type, and therefore fertile.

Probably the most important instance of secondary homothallism occurs in the cultivated mushroom, *Agaricus bisporus*, which, as the specific name implies, usually forms only two spores on the basidium. Each spore must, consequently, be provided with two meiotic products. As the diploid nucleus prior to meiosis was heterozygous at the mating type factor, there are two progeny nuclei of each mating type in the basidium prior to spore formation. Consequently three different genotypes are possible in the spores according to which nuclei migrate together: (A + A) and (a + a), both of which would germinate to produce sterile homokaryons, and (A + a) which would germinate into a fertile heterokaryon: secondary homothallism again because this single progeny spore germinates to give rise to a fertile mycelium. Random segregation of nuclei would give a ratio of 2:1 for heterokaryotic to homokaryotic progeny in any such secondarily homothallic species that packs two compatible nuclei into the same spore. Yet in field collected isolates of *Agaricus bisporus* a significant deviation from this ratio is observed, the heterokaryon being favored. It seems, therefore, that the nuclear migration mechanism may be able to sort nuclei as well as transport them. There are *Coprinus* species that have two-spored basidia, and the spores may have nuclei of compatible mating types, which germinate to give fertile dikaryons in a way analogous to secondarily homothallic *N. tetrasperma* and *A. bisporus*. It has been possible to separate the nuclei from the homothallic dikaryons into monokaryons that were then paired experimentally to study the mating type specificities. Several different *A* and *B* factors were discovered, suggesting that the secondarily homothallic condition originated from a heterothallic one several times in the evolution of the species.

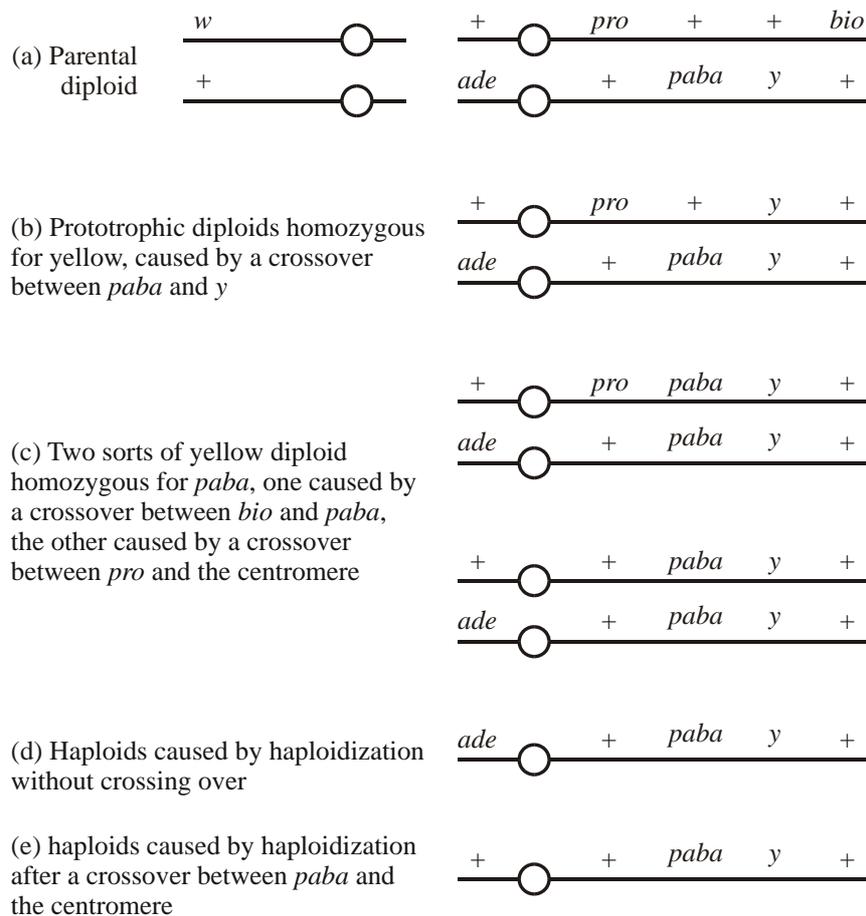## 5.10 Gene segregation during the mitotic division cycle

In the middle of the twentieth century it became evident that meiotic segregations were not the only way of making maps of chromosomes. Mitotic segregations can also be analyzed and are a convenient way of mapping chromosomes. The approach is applicable to any fungus that is normally haploid, although the first step is the selection of diploids that arise spontaneously through nuclear fusion at a rate of about one in every 10^6 or 10^7 mitoses.

The pioneering work was done with the ordinarily haploid filamentous fungus *Aspergillus nidulans*. Selection of diploid strains is a little easier in *Aspergillus* because its conidia are always uninucleate. Consequently, rare diploid conidia can be selected from amongst a large spore population obtained from a heterokaryon by selecting for a heterozygous phenotype. Uninucleate spores cannot be heterokaryotic, so conidia expressing a heterozygous phenotype must contain both homologues of at least one pair of chromosomes, and may be completely diploid. So if you make a heterokaryon between two recessive auxotrophs you would expect that diploid spores would be the only conidia able to grow on minimal medium. Diploid conidia are larger than haploid conidia, being about twice the volume; they also, of course, contain twice the haploid amount of DNA.

This sort of nutritional selection is an automatic method that certainly works efficiently, but it limits the number of nutritional markers that can be used in any experimental crosses. However, an especially useful feature of *A. nidulans* (not true for all fungi) is that the colour of the conidium depends on its own genotype. Consequently, a heterokaryon made between two non-allelic, recessive, spore colour mutants, say white-spore and yellow-spore strains, will produce large numbers of haploid white and yellow conidia together with very occasional sectors of diploid conidia with the wild type dark green colour. Using colour selection leaves open the possibility of having several (unselected) nutritional markers in the cross, but it requires close scrutiny of the cultures.

Nutritional selective methods have been used to isolate diploids from many normally haploid fungi. This includes basidiomycetes such as *Schizophyllum commune* and the agaric *Coprinus cinereus*, but it is especially important in those fungi in which the known life cycle lacks sexual reproduction, and this group includes several commercially important species, including *Aspergillus niger*, *A. oryzae*, *A. flavus*, *Penicillium chrysogenum* and plant pathogens like *P. expansum* and *P. digitatum*.

Diploids are generally sufficiently stable to grow into diploid vegetative colonies, but these do produce rare sectors showing segregation of the originally heterozygous genes. This type of segregation, also based on

(a) Parental diploid

| w | | + | pro | + | + | bio |
| + | | ade | + | paba | y | + |

(b) Prototrophic diploids homozygous for yellow, caused by a crossover between *paba* and *y*

| + | pro | + | y | + |
| ade | + | paba | y | + |

(c) Two sorts of yellow diploid homozygous for *paba*, one caused by a crossover between *bio* and *paba*, the other caused by a crossover between *pro* and the centromere

| + | pro | paba | y | + |
| ade | + | paba | y | + |

| + | + | paba | y | + |
| ade | + | paba | y | + |

(d) Haploids caused by haploidization without crossing over

| ade | + | paba | y | + |

(e) haploids caused by haploidization after a crossover between *paba* and the centromere
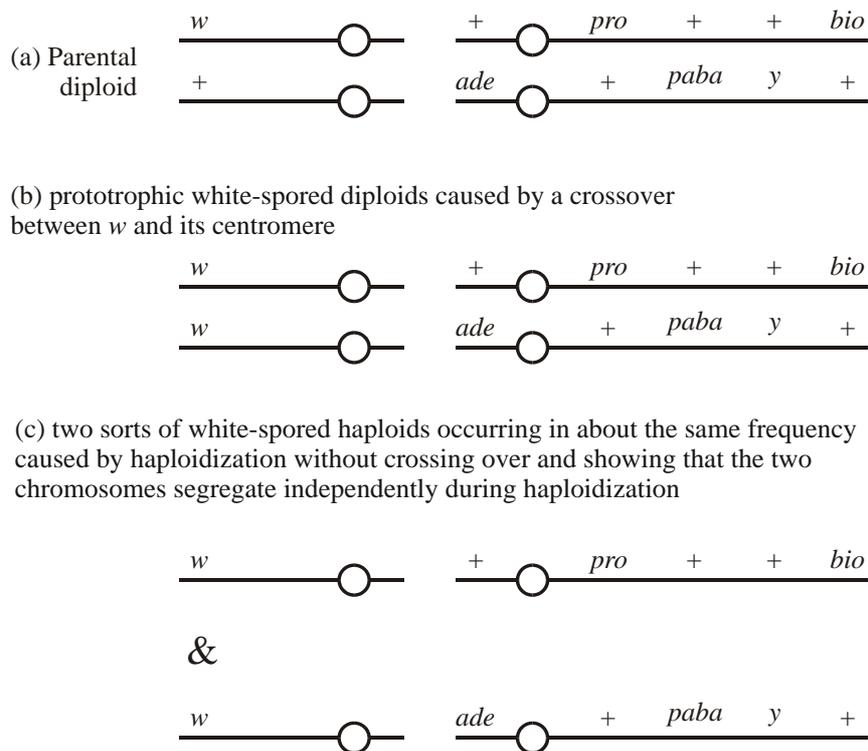
| + | + | paba | y | + |

Fig. 5.6. Yellow-spored mitotic segregants obtained from an experiment with *Aspergillus nidulans* in which the original diploid was heterozygous for both white (*w*) and yellow (*y*) conidia. The parental diploid had the chromosomal constitution shown in Fig. 5.6a, and the crossovers referred to in the other sections of the figure occurred in one chromosome of this genotype before mitotic segregation.

mitotic recombination, can be used for genetic mapping. The method was developed first with *Aspergillus nidulans*, in which segregant sectors of the mycelium could be recognised by the colour of their spores. In *A. nidulans*, mitotic crossing over has a frequency of about two per thousand mitotic divisions and haploidization about one per thousand mitotic divisions. In a reversal of the procedure used for identifying diploid sectors, segregants can be identified by the appearance of yellow or white-spored sectors against the background of dark-green spores of a parental diploid colony heterozygous for recessive colour mutations. Where spore colour cannot be used, other methods for selecting segregants are needed, such as monitoring differential growth between faster-growing haploid sectors and slower-growing diploid sectors. Partially dominant mutants resistant to inhibitors have been used, but suppressors of auxotrophic mutants provide the clearest example. A diploid homozygous for an auxotroph and heterozygous for the (recessive) suppressor cannot itself grow on minimal medium, but can segregate haploids (or homozygous partial diploids) able to grow on minimal medium. These mitotic segregants from the diploid prove to be haploid (produced by a process of regular chromosome loss during successive aberrant mitoses called haploidization), partial diploids (aneuploids stabilised during the chromosome loss sequence) or diploids showing segregation for a few linked genetic markers, and remaining heterozygous for the others. Haploidization is caused by nondisjunction (improper transport of chromosomes to the poles of the division spindle during mitosis) resulting in random chromosome loss over several divisions, so the diploid is reduced to a haploid state through a series of aneuploid intermediates.

We will examine some results from a typical experiment with *Aspergillus nidulans* in which the original diploid was heterozygous for both white (*w*) and yellow (*y*) conidia. These two genes are on different chromosomes, and the *y* chromosome also carried auxotrophic mutations called *ade* (adenine requirement), *pro* (proline requirement), *paba* (requirement for the vitamin *p*-aminobenzoic acid), and *bio* (requirement for the vitamin biotin). The parental diploid had the chromosomal constitution shown in Fig. 5.6a.

Segregants from this diploid were identified on the basis of spore colour: the parental diploid produces dark green conidia, but sectors with yellow spores and sectors with white spores are occasionally found. Amongst the yellow-spored segregants (Fig. 5.6) were strains which were: (i) prototrophic diploids homozygous

Fig. 5.7. White-spored mitotic segregants obtained from an experiment with *Aspergillus nidulans* in which the original diploid was heterozygous for both white (*w*) and yellow (*y*) conidia. The parental diploid had the chromosomal constitution shown in Fig. 5.7a, and the crossovers referred to in the other sections of the figure occurred in one chromosome of this genotype before mitotic segregation.

for yellow, caused by a crossover between *paba* and *y* (Fig. 5.6b); (ii) two sorts of yellow diploid homozygous for *paba*, and therefore auxotrophic for *p*-aminobenzoic acid, one caused by a crossover between *bio* and *paba* in the parental diploid, while the other was caused by a crossover between *pro* and the centromere (Fig. 5.6c); (iii) haploids caused by haploidization without crossing over (Fig. 5.6d); and (iv) haploids caused by haploidization after a crossover; in this case a crossover somewhere between *paba* and the centromere (Fig. 5.6e).

White-spored segregants resulted from homozygosity or haploidization of *w* (Fig. 5.7). White-spored genotypes observed were: (v) prototrophic white-spored diploids caused by a crossover between *w* and the centromere (Fig 5.7b); and (vi) two sorts of white-spored haploids caused by haploidization without crossing over (Fig 5.7c). White-spored haploid segregants requiring proline and biotin were observed in about the same frequency as those requiring adenine and *p*-aminobenzoic acid, showing that the chromosome carrying these auxotrophic markers segregated independently of the white chromosome during the haploidization process.

The yellow diploid segregants show that mitotic crossing over is a reality, so let's see what lessons can be learned from this example. The key to understanding is to remember the crucial differences in chromosome behavior during meiosis and mitosis. In meiosis, homologous chromosomes undergo synaptic pairing, and they take their place on the first division spindle as a bivalent comprising two chromosomes, each divided into two chromatids (this is the four-strand stage as illustrated in Figs 5.2, 5.3 and 5.5). At the first division of meiosis, the so far undivided homologous (that is, maternal and paternal) centromeres are taken to opposite poles of the division spindle (Fig. 5.2). *None of this happens in mitosis*.

In mitosis homologous chromosomes do not line up with one another, so there is no synapsis. The fact that recombinant diploid segregants can be obtained from mitotic crossing over indicates that occasional exchanges occur between homologous chromosomes during mitosis. Data obtained from other experiments have demonstrated that mitotic crossing over is a reciprocal event, that is, the recombination results in two homologous chromosomes with reciprocally recombinant arms (Fig. 5.8), but it is clearly extremely rare. In fact, mitotic crossovers are too rare for double exchanges to be a problem in genetic analysis. Mitotic crossing over can be visualized as very similar to meiotic crossing over, but the consequences in terms of the genotypes of progeny nuclei differs because chromosome segregation differs between meiosis and mitosis. If a crossover takes place in mitosis the two chromosomes involved do not stay together (as they do in meiosis), but they separate

Meiosis
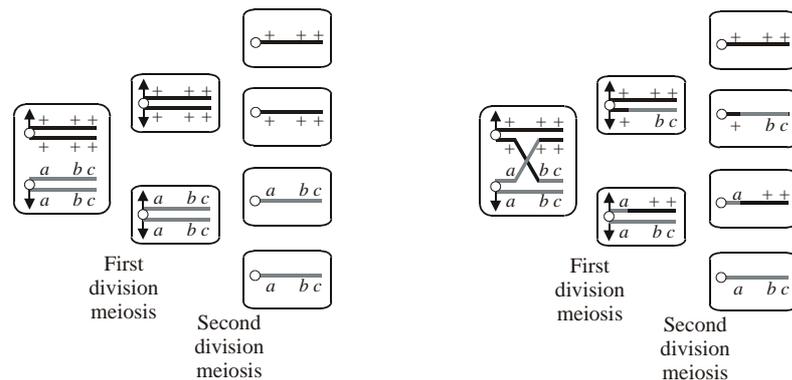
Mitosis
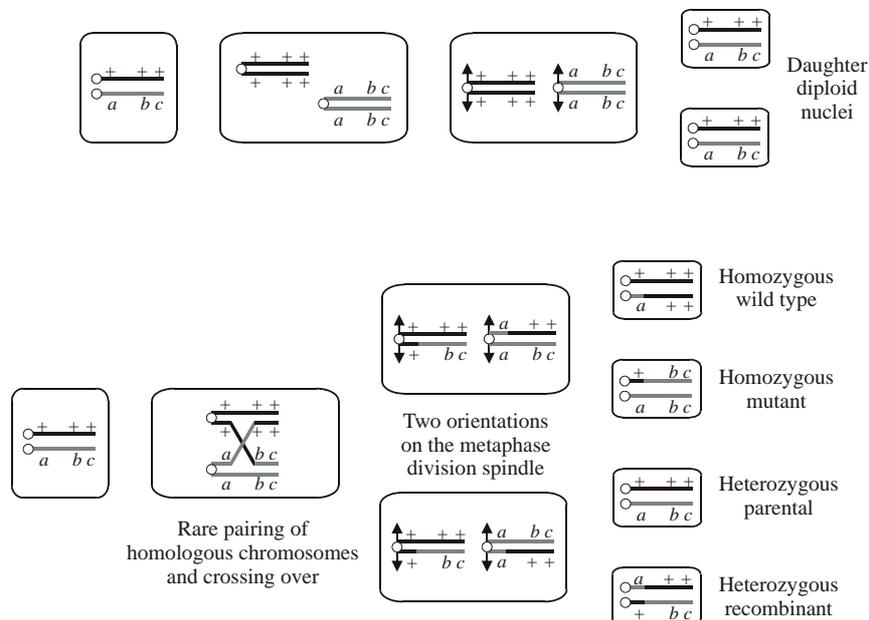
Fig. 5.8. Comparison of the segregation mechanisms of meiosis and mitosis. At the top we show meiosis, on the left without recombination, on the right incorporating a crossover event. These are the perfectly normal segregation processes, also illustrated in Fig. 5.3. Mitosis, with and without a crossover, is shown in the bottom part of the figure. Mitosis starts with replication of the parental chromosomes, but the two homologues do not normally associate with one another. Subsequently, the replicated chromosomes align on the mitotic division spindle independently, and the rule is that one daughter chromatid of each replicated chromosome segregates into each daughter nucleus, so that the two daughter diploid nuclei have the same genotype as the parent. Very rarely, the replicated chromosomes do associate with one another sufficiently closely for a crossover to occur. This results in two of the daughter chromatids being recombinant. However, the recombinant replicated chromosomes still independently align on the mitotic division spindle, and still follow the rule that one daughter chromatid of each replicated chromosome segregates into each daughter nucleus. Because of the crossover, there are two possible orientations on the mitotic division spindle. One produces two diploid daughter nuclei that are homozygous from the point of the crossover to the end of the chromosome arm (one homozygous mutant, one homozygous wild type). The alternative orientation produces two heterozygous diploid daughter nuclei, but one contains the two parental chromatids, and is exactly the same as the parental nucleus, whereas the other receives the two recombinant chromatids.

and reach the equator of the division spindle independently. The two (homologous) chromosomes, which are, of course, divided into two daughter chromatids, then behave independently. In meiosis, the two reciprocally recombinant chromatids ***must*** end up in different haploid daughter nuclei (Fig. 5.8).

But mitosis produces ***diploid*** daughter nuclei by sending one daughter chromatid of ***each*** homologous chromosome to each pole of the division spindle. Providing the rule that daughter chromatids must go to opposite poles of the division spindle is upheld, there is no other constraint. The daughter chromatids of a pair of homologous chromosomes segregate independently of each other. Consequently, following a mitotic crossover, the two reciprocally recombinant chromatids may pass to opposite poles at the subsequent anaphase stage of mitosis, alternatively, and with equal probability; they may go to the same pole. The importance of this is that in the former case (where a recombinant chromatid is accompanied by a parental chromatid) all markers between the crossover and the end of the chromosome will become homozygous (Fig. 5.8).

For practical analysis, the rule is that in diploid segregants homozygosis for one gene will always be accompanied by homozygosity of any genes distal to it in the same chromosome arm, but not necessarily by homozygosity of genes more proximal to it. This general characteristic of mitotic segregation is what allows us to deduce the genetic map of the segment of chromosome involved, and is illustrated in the yellow diploid segregants in Fig. 5.6. Homozygosity for *y* was invariably accompanied by homozygosity for the wild type allele of *bio*. This fact places the *bio* gene distal to *y* in the same arm of the chromosome. Similarly, homozygosity for *paba* was frequent but not invariable in diploids homozygous for *y*, while homozygosity of *pro* was even less frequent in the yellow homozygotes. This pattern indicates that *paba* and *pro* were proximal to *y* in the same arm; that is, on the centromere side of *y*, with *pro* the nearer to the centromere. Noting that co-segregation of *ade* and *paba* in yellow haploids suggests that the two genes are linked, the absence of yellow diploid segregants homozygous for *ade* allows us to infer that this gene may be in the other arm of the chromosome (other data confirm that homozygosis of one arm of a chromosome occurs independently of homozygosity of the other arm). The frequencies of the different sorts of homozygous diploid segregants are a measure of relative map distance between the genes; but it must be stressed that mitotic recombination is rare, so it is not easy to assemble a sample of independent segregants large enough to make frequency measurements reliable. Nevertheless, even a few segregants can give absolute guidance about gene order relative to the end of the chromosome arm.

In most organisms, the analysis of meiotic products is usually the easiest way of mapping the genome. However, use of mitotic segregations for genetic analysis does offer some worthwhile advantages over meiotic analysis to the experimenter. Relatively few segregants can provide a considerable amount of information about relative positions of genes on the chromosome. Even one diploid segregant in which linked genes *a* and *b* become homozygous simultaneously provides evidence that they are in the same chromosome arm, and a second segregant in which *a* becomes homozygous alone shows almost certainly that *a* is distal and *b* proximal with respect to the centromere.

Meiotic and mitotic linkage maps show the same gene orders but the spacing between the genes differs, implying different distributions of crossovers in mitosis and meiosis. The overwhelming advantage of mitotic analysis, though, is in the formation of haploids. Because mitotic recombination is so rare, genes on the same chromosome show complete linkage during haploidization. Genes reassort freely if they are not on the same chromosome. Thus, linkage group assignments can be made far more efficiently than is possible in meiotic analysis.

Application of the approach to other organisms depends on the stability of the diploid state. This varies enormously between species. *Verticillium albo-atrum* seems to hold the record for instability: a random sample of 540 conidia giving thirty-seven segregants. *Coprinus radiatus* diploids are also unstable, though the closely related *C. cinereus* has segregant frequencies of around 10^{-4}. Diploid segregants due to mitotic crossing over occur with similar frequency (range 10^{-3}-10^{-4}) in *Saccharomyces cerevisiae* in which diploidy is a normal part of the life cycle. Diploids of *Ustilago maydis* seem to be completely stable, but mitotic crossing-over is inducible by ultraviolet light. X-rays, nitrous acid, mitomycin C, fluorodeoxyuridine and the amino acid analogue *p*-fluorophenylalanine have also been used to increase frequencies of segregation from diploids. The common link between these agents seems to be that they cause damage to DNA that induces repair mechanisms which themselves initiate processes leading to mitotic crossing-over and haploidization.

We have described a number of separate events occurring during vegetative fungal growth that might be arranged into a sequence. The fusion of genetically different haploid nuclei in a heterokaryon followed by mitotic crossing-over and completed by haploidization, is a sequence termed the parasexual cycle. On the face of it, the parasexual cycle has much the same effect as the sexual cycle by reassorting and recombining genes, thereby increasing genetic variation within the species. A plausible argument can be made that the parasexual cycle could be an alternative to sex in imperfect fungi, but there is not much clear evidence for this. Indeed, not a great deal of practical use has been made of the parasexual cycle in the laboratory, even though several commercial processes depend on imperfect fungi like *Penicillium chrysogenum*. Ironically, the technique has found its most extensive application in human genetics. A very large proportion of the gene assignments to human chromosomes were made using the analogous cycle: mouse + human cell forming a hybrid fusion cell which suffers successive loss of chromosomes during subsequent mitoses. Eventually, aneuploid cell lines,

sufficiently stable for genetic and cytogenetic characterization are formed and co-segregation of genes reveals linkage.

5.11 Cytoplasmic segregations: mitochondria, plasmids, viruses and prions
Our focus so far has been the segregation of nuclear genes, but there are a number of cytoplasmic genetic elements which affect the fungal phenotype and which depend upon some of the features of the sexual cycle for their transmission. Chief among these are the mitochondria. Mitochondrial genomes are independent of and quite distinct from the nuclear genome. Mutations in mitochondrial genes result in particular phenotypes in both *Neurospora crassa* and *Saccharomyces cerevisiae*, which arise through loss of mitochondrial function. The mitochondrial genome contains genes for mitochondrial ribosomal RNAs and at least some of the proteins of the respiratory chain. In other words, the organelle genome specifies some of the organelle proteins, but not all of them. The rest of the organelle proteins are coded by nuclear genes, synthesized in the cytoplasm, and then transported into the mitochondrion.

Genetic maps of mitochondrial genomes, and physical maps derived from restriction analysis, are circular. However, in many cases the organelle will have linear versions of the genome coexisting with the circular one. *S. cerevisiae* can have over 100 genomes per mitochondrion, corresponding to about 6500 in each cell. Oddly enough, when segregation of organelle genes is followed in genetic crosses, the segregation patterns are consistent with there being only one copy of the mitochondrial genome in the cell. The fact that this is clearly not the case indicates that we do not fully understand how organelle genomes are transmitted from parent to offspring.

Vertical mitochondrial transmission (that is, from one generation to the next) can depend upon whether or not the fungal strains concerned in a cross show differentiation into 'males' and 'females'. In *Neurospora crassa*, phenotypes controlled by mitochondrial genes are generally transmitted through the female, that is the protoperithecial parent. Whether this is because of the smaller volume of cytoplasm present in a microconidium (as the male) is not certain. Yet paternal and even recombinant mitochondria are sometimes found in the sexual progeny. For example, in a cross between two strains of *N. intermedia*, maternal, paternal and recombinant mitochondrial DNA have been found in the progeny.

Horizontal transmission (between individuals of the same generation) occurs as a result of hyphal fusion. Although complementation and recombination can be detected between mitochondrial genomes, mycelia containing genetically different mitochondria (called heteroplasmons or heteroplasmic mixtures) tend to segregate the different mitochondria into different cells. Cytoplasmic segregation is a general feature of organelle genomes, for example in the formation of asexual conidia.

Uniparental inheritance of mitochondrial phenotypes has been observed in yeast, which is isogamous (does not show a male/female differentiation). Again, the mechanism is unknown, but even in the usual biparental inheritance pattern, mitochondrial genomes can segregate in association with mitosis and the consequential budding. End buds frequently contain one parental mitochondrial DNA, while later buds are heteroplasmic or contain recombinant mitochondrial DNA.

In filamentous fungi, mitochondria are not thought to be closely associated with the mitotic spindle, so vegetative segregation may simply be a matter of random physical sorting, though nuclear and mitochondrial genes do influence mitochondrial genome transmission and it is also affected by membrane chemistry. In some ascomycetes sub-cultured for a long time in the laboratory, altered mitochondrial DNAs due to molecular rearrangements have been associated with modifications in mycelial growth in *N. crassa* and *N. intermedia*, and in the cellular growth in yeast.

In *Schizophyllum commune* mitochondria do not migrate with nuclei during dikaryosis. Mitochondrial inheritance has been studied in matings of *S. commune*, *Agaricus bitorquis*, *A. brunnescens*, *Coprinus cinereus*, *Lentinula edodes*, *Pleurotus ostreatus*, phytophathogenic *Armillaria* species and *Ustilago violacea*, and in all these cases, plasmogamy may result in the production of mycelial colonies composed of sectors containing different mitochondrial DNAs (mitochondrial mosaics). In *Agaricus bitorquis*, *A. bisporus*, *Armillaria bulbosa*, *P. ostreatus* and *U. violacea*, analysis of dikaryons gave evidence for mitochondrial mixing, sometimes followed by recombination between mitochondrial genomes; recombinant mitochondrial DNAs have also been recovered from dikaryons and dikaryotic protoplasts of *C. cinereus*.

Mitochondrial DNA (mtDNA) of yeast usually makes up 18% of the total DNA, but it has a distinctive very high (82%) AT content so is relatively easy to separate from chromosomal DNA. The mtDNA is circular, of 25 nm circumference, and comprises about 7.5×10^5 base pairs. In yeast, mtDNA codes for three of seven polypeptides of the cytochrome *c* complex (the rest derive from nuclear genes), four polypeptide components of a mitochondrial ATPase, and one component of cytochrome *b*. Mutations in these genes can produce recognizable respiratory deficiency phenotypes (for example, *petite* in *Saccharomyces cerevisiae*, *poky* in *Neurospora crassa*) and thereby provide mitochondrial mutants. Also, although chromosomal genes code mitochondrial ribosomal proteins, mtDNA determines mitochondrial ribosomal RNA (rRNA) and transfer RNAs (tRNAs). Mitochondrial ribosomes are similar in size to prokaryotic ribosomes and share some other prokaryotic properties; in particular, protein synthesis on mitochondrial ribosomes is inhibited by chloramphenicol,

erythromycin and several other antibacterials that have no effect on cytoplasmic (eukaryotic) ribosomes. Consequently, another kind of mutant phenotype due to mitochondrial mutation is resistance to inhibition by mitochondria-specific drugs. Mitochondrial gene sequences are also similar to equivalent genes in prokaryotes. These features encouraged the endosymbiont theory of mitochondrial origin, which envisages mitochondria to be relics of ancient bacteria-like organisms that formed a symbiotic association that resulted in the ancestral 'eukaryotic' cell.

The basic procedure for making mitochondrial crosses involves making heterokaryons (diploids in yeast) between haploids carrying the mitochondrial markers. Heterokaryosis may be forced with complementary nutritional (nuclear-gene) mutations. After some vegetative growth of the heterokaryon or diploid, diploid daughter cells (of yeast) or spores or hyphal fragments (of filamentous fungi) are plated out and the resulting colonies are scored for the mitochondrial markers present in the original haploids. Parental and recombinant clones are usually found, and in relative frequencies that depend very much on the nature of the haploid parents. It is also possible to use the heterokaryons for complementation analysis. If the constituent haploids carry mutations in different mitochondrial genes they are expected to complement each other in heterokaryons formed between them.

Because the mtDNA is circular, viable recombinants can only result from even numbers of crossovers between DNA molecules. The highest recombination frequency between distant markers is about 25%, and (again, because of the circularity) most pairs of markers tend to show about the same frequency. Nevertheless, multiple-point crosses can effectively define the order of the markers round the circle. Of course, although this sort of recombination *in vivo* may remain important to the organism in nature, it has been supplanted by direct physical analysis of the DNA molecule in laboratory studies.

An increasingly important aspect of mitochondrial transmission is their content of plasmids. Isolates of *Neurospora* from nature commonly contain both linear and circular mitochondrial plasmids. Most are cryptic (that is, neutral) passengers, but some linear plasmids (notably of *Podospora anserina*) insert into mitochondrial DNA and cause mycelial senescence. Most linear plasmids exhibit typical virus characteristics as far as structure, replication and function are concerned and even plasmid-free strains may contain plasmid remnants integrated into their mitochondrial DNA. Plasmid DNA sequences generally encode an RNA polymerase and DNA polymerase, or reverse transcriptase which are used to maintain and propagate the plasmid.

However, plasmid DNA is responsible for the killer phenomenon in the yeast *Kluyveromyces lactis* by coding for a killer toxin, which kills cells lacking the plasmid (cells hosting the killer plasmid are immune to the toxin). These plasmids reside in the cytoplasm and have an expression system independent of both nucleus and mitochondrion. *K. lactis* plasmids can be transferred to other yeasts (including *Saccharomyces cerevisiae*), conferring the killer/immunity phenotype. This shows that the plasmids are autonomous replicons, which can be expressed, in a wide range of host yeasts. The *K. lactis* killer plasmid toxin is chemically and functionally different from a killer toxin produced in *S. cerevisiae*, which is encoded by a double-stranded RNA (dsRNA) virus.

Virus-like particles (VLPs) have been observed in many fungi. They are very similar in appearance to small spherical RNA viruses that have been found in filamentous fungi, but there is little evidence that these particles are effective in hypha-to-hypha infection. Many of the observed VLPs are presumably degenerate or defective viruses that can only be transmitted by hyphal fusions. However, viruses of *Agaricus bisporus* require different RNA molecules to produce infective particles as though some are defective viruses and others are helper viruses. Eukaryotic cells do not normally replicate RNA, so these functions are presumably demanding for the virus genome so that different classes of virus have evolved, each performing a special, complementary, function.

S. cerevisiae also harbors five retrovirus-like elements, Ty1 to Ty5 as transposons able to integrate into the nuclear genome by targeting particular chromatin structures. The first cytoplasmic plasmid to be observed is the so-called two-micron DNA of *Saccharomyces* (= Ty1). The name refers to the contour length of the circular DNA molecules that has a base composition similar to nuclear DNA and quite different from mtDNA. There can be 50 to 100 two-micron DNA molecules per diploid cell, amounting to something like 3% of the nuclear DNA. The two-micron DNA molecules are transmitted in nonmendelian fashion, independently of both nuclei and mitochondria. The two-micron circular DNA carries inverted repeat sequences at either end of two different unique sequence segments; this structure implies that it inserts itself as a whole into the yeast chromosome.

So far our discussion has dealt with nucleic acid molecules that encode features segregating in a nonmendelian manner. In the final decade of the twentieth century, however, great attention was given (and continues to be given) to a proteinaceous hereditary element, called a prion protein. The attention devoted to prions derives from their ability to cause diseases in mammals: scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and in humans, kuru and, most importantly, new variant Creutzfeldt-Jakob disease (nvCJD). In these cases, the pathogenic agent is a variant of a normal membrane protein (the prion protein) that is encoded in the mammalian genome. The variant prion protein folds abnormally and causes normal prion proteins to fold abnormally so that the proteins aggregate in the central nervous system and cause the encephalopathy. Fungi also have prion proteins.

We have already referred briefly (section 2.3) to the infectious *het-s* phenotype in *Podospora anserina*, which may be a prion protein. An even better candidate is the *PSI*[+] form of the Sup35p protein in *Saccharomyces cerevisiae*. Sup35p is an essential yeast protein involved in the termination of translation. In the *PSI*[+] state, Sup35p adopts a structural conformation that causes it to direct the refolding of native molecules into a form that can aggregate into filaments of discarded nonfunctional protein. This depletes the cytoplasm of functional translation terminator and results in translation errors. This is the *PSI*[+] phenotype, which is inherited by daughter cells following budding and is infectious following cell fusion, in which case it propagates by autocatalytic conversion of the normal form of the protein. The part of the Sup35p protein that makes it a prion (the prion determining domain) is a glutamine/asparagine-rich amino-terminal region that contains several oligopeptide repeats. Removal of these repeats eliminates the ability of Sup35p to propagate *PSI*[+], and expanding the repeat region increases the spontaneous occurrence of *PSI*[+]. Although deleting the analogous repeats from BSE prion protein does not prevent prion propagation and transmission in experimental mice, expansion of the repeat region does increase the spontaneous appearance of spongiform encephalopathies by several orders of magnitude in humans.

It seems very likely that it is the oligopeptide repeats that gives the prion protein the intrinsic tendency to acquire a conformation that enables the protein to refold and effectively polymerize with sister molecules. Database searches for regions with amino acid content comparable to the yeast prions has revealed numerous such domains in eukaryotes, but these are, significantly, lacking from prokaryotes. We need urgently to know whether these other eukaryotic proteins can also behave as elements of protein based inheritance like prions. Several other human diseases are associated with (and might be caused by) protein misfolding: Huntingdon's Chorea, ataxias, Parkinson's disease, Alzheimer's disease. The proteins involved also have repeating motifs. Further analysis of fungal prions may help in understanding such diseases, but beyond this, there is the question of prion biology. Recent work shows that the yeast Sup35p prion provides a selective advantage under adverse conditions, possibly by producing phenotypic variants. These arise because the SUP35 gene encodes translation release factor 3, but in the *PSI*[+] state, *PSI*[+] aggregation results in inefficient termination of stop codons. This might imply that the prion state is retained because it aids evolution.

Recent publications and websites worth a visit

Davis, R. H. (2000). *Neurospora: Contributions of a Model Organism*. Oxford University Press: Oxford, UK.
Fungal Genetics Stock Center website at http://www.fgsc.net/methods.
Martinelli, S. D. & Kinghorn, J. R. (1994). *Aspergillus: 50 Years on*. Elsevier: Amsterdam.
Pál, C. (2001). Yeast prions and evolvability. *Trends in Genetics* **17**, 167-169.
Perkins, D. D., Radford, A. & Sachs, M. S. (2000). *The Neurospora Compendium: Chromosomal Loci.* cademic Press: Orlando, FLA, U.S.A.
Silar, P. & Daboussi, M. –J. (1999). Non-conventional infectious elements in filamentous fungi. *Trends in Genetics* **15**, 141-145.
Strathern, J.N., Jones, E. W. & Broach, J. R. (1981). *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press: New York, U.S.A.
True, H. L. & Lindquist, S. L. (2000). A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* **407**, 477-483.
Tuite, M. F. & Lindquist, S. L. (1996). Maintenance and inheritance of yeast prions. *Trends in Genetics* **12**, 467-471.

Historical publications worth knowing about

Clutterbuck, A. J. (1974). *Aspergillus*. In *Handbook of Genetics*, **vol 1** (ed. R. C. King, p. 447-510. Plenum Press: New York.
Esser, K. (1974). *Podospora anserina*. In *Handbook of Genetics*, **vol 1** (ed. R. C. King, p. 531-551. Plenum Press: New York.
Gans, M. & Prud'homme, N. (1976). Chromosome maps of *Coprinus radiatus*. In *Handbook of Biochemistry*, 3rd edn, *Section B, Nucleic Acids*, **vol. 2**, (ed G. D. Fasman), pp. 762-764. CRC Press: Cleveland, Ohio.
Moore, D. (1967). Four new linkage groups in *Coprinus lagopus*. *Genetical Research* **9**, 331-342.
Mortimer, R. K. & Hawthorn, D. C. (1966). Genetic mapping in *Saccharomyces*. *Genetics* **53**, 165-173.
Radford, A. (1976). Chromosome maps of *Neurospora crassa*. In *Handbook of Biochemistry*, 3rd edn, *Section B, Nucleic Acids*, **vol. 2**, (ed G. D. Fasman), pp. 739-761. CRC Press: Cleveland, Ohio.
Sherman, F. & Lawrence, C. W. (1974). *Saccharomyces*. In *Handbook of Genetics*, **vol 1** (ed. R. C. King, p. 359-393. Plenum Press: New York.