A Karyological Study on *Peziza subumbrina* Boud., with Special Reference to a Heteromorphic Pair of Chromosomes

By

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(With Plates IX–XII and 1 Text-figure)

Recently members of the Ascomycetes, especially the genus *Neurospora,* have been genetically dealt with by some investigators, with regard to the differentiation of sex in connection with the mode of reduction division (*cf.* Dodge '27, '29, Wilcox '28, Lindegren '32). It is now generally admitted that heterothallic fungi are of heterozygous nature regarding the 'sex factors' and that segregation takes place at meiotic division of the ascus nucleus.

The existence of a heteromorphic pair of meiotic chromosomes in the present material, *Peziza subumbrina* Boud., a member of the Discomycetes, which is dealt with in this article, will be of special interest in the consideration of the mode of reduction division which has been a subject of lively discussion among cytologists dealing with the Ascomycetes (*cf.* Tischler '21–'22, Gäumann '26, Kniep '28, etc.), and also on account of a possibility that it may offer a key to the interpretation of sexual differentiation in fungi, because it is a commonly accepted fact that the Ascomycetes in general exhibits an alternation of generations which does not differ in its essential nature from that of the higher plants.

Since no report has been previously made on such hetero-chromosomes in fungi, detailed observations were concentrated on this point in the present study.

Material and Methods

The material under investigation, *P. subumbrina,*[1] was collected at the vicinity of Sapporo during the summer and autumn of 1932 and 1933.

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1) The writers are indebted to Mr. S. Imai, a mycologist of the Botanical Institute, Faculty of Agriculture of this University, for the identification of the species.

Young ascocarps were cut in small pieces and fixed in the field at the daytime or in the laboratory at midnight. The fixatives employed are ALLEN's modified solution of BOUIN's fluid or FEULGEN's fluid containing no alcohol. As soon as the material was thrown into the fixatives, air was removed with the aid of an air-pump to secure prompt infiltration of the fixing fluid. The material was embedded in paraffin in the usual way; sections were cut from 3 to 8 μ in thickness and stained with HEIDENHAIN's iron-alum haematoxylin or with NEWTON's gentian-violet. For critical observations on the chromosomes, the latter proved to give a better result. Before staining, the slides were immersed for 3 or 4 hours in a mixed solution of pepsin and 0.2% hydrochloric acid at a temperature of 38°C.

Descriptions

Ascus-formation. The two associated nuclei travel in pair up the ascogenous hyphae and the latter bends over to form a hook, as described by several investigators in other fnngi (Fig. 1). The two nuclei begin then to divide simultaneously (Fig. 2) and four chromosomes are sometimes countable at metaphase in each nucleus. In one of the two nuclei the chromosomes are all small and oval-shaped, while in the other there is a large rod-shaped chromosome besides the three small ones. At anaphase eight chromosomes can be seen (Fig. 16). As soon as the division is completed a terminal uninucleate and a penultimate binucleate cell are cut off (Fig. 3), and the latter fuse together into a diploid nucleus, the primary ascus nucleus (Figs. 4, 5 and 6). The mechanism of this nuclear fusion has not been clarified but it seems to take place in the form of chromosomes. The primary ascus nucleus becomes oval in shape, with faintly stained nuclear network. At the same time, two nucleoli in it fuse together, giving rise to a large one, which is approximately twice as large as the early one.

The ascus then grows and the definitive nucleus increases in size, somewhat elongating longitudinally, and takes a position in the upper portion of the ascus. The mature nucleus, which is almost elliptical in shape, has a single deeply stained nucleolus. The reticulum stains faintly at this stage, and its distinct structure can not be traced (Fig. 7).

First division. The nucleus at prophase attains its maximum size and sometimes within the nucleolus a single or several small vacuoles are seen (Fig. 7). In some cases, the single vacuole attains a comparatively large size, occupying most of the body of the nucleolus, leaving only a thin dark-stained boundry.

Then synizesis follows, the spireme contracting together close to the

nuclear membrane and usually the spireme attaches to the nucleolus (Fig. 8). As the nucleus passes out of the synizetic contraction, the spireme becomes distributed over the nuclear cavity and more or less thicker (Fig. 9). At a later stage the spireme and the nucleolus appear considerably to decrease their chromaticity for a while (Fig. 11). The spireme then becomes granular in appearance and its dual nature is manifested throughout it (Fig. 12).

Shortly thereafter the spireme breaks up to form four threads which are twisted with each other (Fig. 13). Then the threads contract to a mass around the nucleolus and the four gemini are first distinguished at a later stage (Figs. 14 and 15). At these stages two centrosomes appear on the nuclear membrane and between them are formed the spindle fibers which seem to be of intranuclear origin (Fig. 15).

After the contraction the chromosomes extend within the nuclear cavity and enter into metaphase. The diakinesis has not been observed.

It may be inserted here that extrusion of chromatin bodies is a remarkable event of the prophase stages, as previously noted by several investigators in other fungi (*e.g.* CARRUTHERS '11, BAGCHEE '25, WAKAYAMA '30). It occurs also at the two nuclei before fusion (Figs. 4 and 5), but is more remarkable at meiotic prophase (Figs. 9–11). The chromatin bodies are extruded both from the nucleolus and from the ends of the spireme which attach to the nuclear membrane. It was often observed that a chromatin body extruded into the cytoplasm is connected with the nuclear membrane by a fine thread (Fig. 10). Probably this may have resulted from the migration of the nucleus through the cytoplasm. A clear zone around the extruded bodies, as observed in higher plants, was visible here too. These bodies seem to decompose gradually and finally disappear at metaphase.

At metaphase, four gemini are clearly distinguished; three are equal pairs of small chromosomes and one is a pair consisting of a large rod-shaped chromosome and a small one (Fig. 18). In the figures of Plate II, the large component of the heteromorphic pair is represented by a letter '*a*' and the small partner by '*a*''. Figs. 19–21 represent early anaphase, where the halves of each bivalent are beginning to separate from each other. Very often in one of the three equal pairs, the second longitudinal splitting occurs as early as at this stage, thus rendering the chromosome number as many as ten. Figs. 22–25 represent such a condition. As the chromosomes advance towards the poles, the number of split dyads seems to increase; in Fig. 26 probably two equal pairs, and in Fig. 29, all the

members are subjected to the second splitting. Often the long chromosome (*a*) presents a V-shaped appearance at these stages (Fig. 26).

It is a rather striking fact that the heteromorphic pair divides very rarely after the equational manner, as represented in Fig. 28. In it each two of the split halves of dyads (a_1 and a_1', a_2 and a_2') are shown to pass towards each pole. In Fig. 30 (*cf.* also Fig. 45) the split halves of the large component are seen lagging in the plate.

As will been seen in these figures and several photomicrographs of Plate XII representing metaphase and anaphase of the first division, the heteromorphic pair differs from the other equal pairs in its mechanical properties, *i.e.*, in that it varies to a certain extent in size and staining capacity, its contour is often irregular and it lies off the plate.

As the chromosomes reach the poles they contract and reconstruct the daughter nuclei (Figs. 35 and 36). They pass into the interkinesis and are considerably smaller than the primary ascus nucleus.

The nucleolus persists throughout the process of division, without showing any sign of degeneration. At the formation of the daughter nuclei, it is left in the cytoplasm (Fig. 36) and finally disappears. The spindle becomes a thick bundle, increasing its length, which persists until the nuclear membrane formation of the daughter nuclei (Fig. 42), as described by BROWN ('11) in *Lachnea scutellata*. Sometimes the remainder of the bundle was observed to lie in the daughter nucleus (Fig. 43).

Second division. After interkinesis the second division begins but passes rapidly, rendering observations on its details very difficult. There is no apparent synizesis. At metaphase four chromosomes, and at anaphase eight chromosomes, are countable on the spindle (Figs. 31 and 37). In Fig. 31, the large rod-shaped chromosome is clearly identified in one of the daughter nuclei. Fig. 32 represents an anaphasic nucleus, in which the large chromosome is going to divide, taking a V-shape. The behavior of the chromosomes in separation is somewhat irregular and each chromosome often shows splitting, separating in two's as in the first division.

During the second division, the spindles are longitudinally or slightly obliquely placed in the ascus, thus leading to the formation of four nuclei in a row (Figs. 37 and 47).

Third division. At the onset of the third division, the chromatin of each nucleus forms a delicate spireme which later tends to gather towards one side of the nuclear cavity, giving an appearance like the synizetic stage of the first division (Fig. 41). The spindle is formed in an oblique or transverse direction with regard to the long axis of the ascus, and the

chromosomes scatter on it (Fig. 38). Very often a chromosome-like fragment of the spindle bundle still persists in the nuclear cavity, imposing certain difficulties on the observation of chromosomes at this stage. In favorable cases, however, four chromosomes are clearly distinguished. Fig. 33 represents a metaphase plate of one nucleus in which the long chromosome (*a*) is easily identified. Fig. 34 shows four chromosomes at anaphase, all of which are small, thus indicating that the nucleus has not received the long chromosome. To the writers' deep regret, however, they could not obtain any favorable case in which the distribution of the heteromorphic members is traceable in all the four nuclei in an ascus. It is clear, however, that there is no such numerical diminution of chromosomes at this division as brachymeiosis demands; on the contrary, some half chromosomes often show the second splitting, as in the first and the second division, thus the chromosome number amounts to more than eight. The chromosomes having reached each pole they contract (Fig. 39) and finaly form the eight daughter nuclei.

The membrane of a spore is formed by the astral radiation originating from a centrosome which lies at the top of the pear-shaped nucleus. Thus the spores are uninucleate (Fig. 40).

Discussion

The present finding demonstrating the existence of a heteromorphic pair of chromosomes in *Peziza subumbrina* will be of special interest in view of the mode of reduction and its bearing upon the segregation of genes for heterothallism in the Ascomycetes. These two points constitute the subject of the following discussion.

(i) *The mode of reduction.* From the foregoing description, it is obvious that in the present material, there is only one reduction division. This was clearly indicated by the behavior of the heteromorphic pair which comprises a large rod-like chromosome and a small dot-like one. Usually they divide in a reductional manner in the first division and each half of the geminus undergoes the subsequent two divisions of equational nature. Very rarely however they divide equationally in the first division, each two half dyads of the geminus passing to each pole at anaphase. In these cases the second mitosis will be most probably of a reductional nature. Owing to the very rare occurrence of this mode of division, the present observation failed actually to demonstrate it in the second mitosis, but at the same time it may be added that the writers have met with no case of the third division presenting any sign of numerical reduction of chromo-

somes.

These two modes of reduction may be similarly expected for the other three equal pairs, though the actual demonstration of it was impossible. The frequent occurrence of split half dyads of certain gemini at very early anaphase may have been due to the equational first division. But at the same time an alternative interpretation that it merely indicates the earlier accomplishment of the second splitting in particular chromosomes than the others is equally valid; in reality during migration of chromosomes towards the poles, the split chromosomes were observed to increase in number, thus finally resulting in the appearance of eight chromosomes at each pole. This apparent increase in 'chromosome number' during the mitosis, together with the minute size of the chromosomes, imposes serious difficulties on the determination of the chromosome number and consequently the mode of reduction. It may then be quite probable that material offering no clue for identifying the individual chromosomes some anaphase figures (such as Fig. 28) may readily lead one to count the chromosomes in double number and to conclude that the process of 'brachymeiosis' has occurred. The writers fear that some earlier observations do not exclude the possibility of this.

Recent critical studies, both cytological and morphological, on the Ascomycetes tend to point to an intimate connection between the number of fusions ('fertilization'), that is, double or single, and the mode of reduction division. This is concerned with the chromosomal organization of the spore mother-cell; the double fusion of nuclei in the life-cycle, no matter how the first fusion takes place,[1] results in the formation of the ascus of a tetraploid nature and as a corollary there should be 'double reduction' or brachymeiosis in the ascus; on the other hand, in cases of the complete absence of the first fusion, single reduction division will suffice to produce haploid spore nuclei. An instructive instance of this is one offered by TANDY ('27) who reports that in *Pyronema domesticum* only some of the sexual nuclei fuse in the ascogonium, the others remaining as unpaired nuclei. These unpaired nuclei pass with the fusion nuclei

1) In the Ascomycetes, the following several modes of the first nuclear fusion have been known: normal fusion of sexual nuclei in the ascogonium (*e. g.* HARPER 1895–1905; CLAUSSEN '05; BLACKMAN & FRASER '05; GWYNNE-VAUGHAN & WILLIAMSON '31, '32, '34, etc.), a fusion of ascogonial nuclei in the absence of the functional antheridium (*e. g.* FRASER '07; FRASER & BOOKS '09; GWYNNE-VAUGHAN & WILLIAMSON '30, etc.), and a fusion of vegetative nuclei in the absence of defined sexual organs (*e. g.* FRASER '08; CARRUTHERS '11, etc.).

into the ascogenous hyphae, so that the latter bears both haploid and diploid nuclei, and gives rise after the second fusion in the ascus hook to the ascus which contains either a diploid or a tetraploid nucleus. In diploid asci, reduction division takes place in a 'single' form, but in tetraploid asci it takes place in a 'double' form.

The present data for *P. subumbrina* present a clear instance of a single reduction division. Though the whole life-cycle of this fungus has not been investigated, the absence of the first fusion will be obvious, so far the chromosomal situation indicates. It will be then concluded that *Peziza subumbrina* is a species characterized by a more simplified life-cycle than others in which the fusion takes place two times.

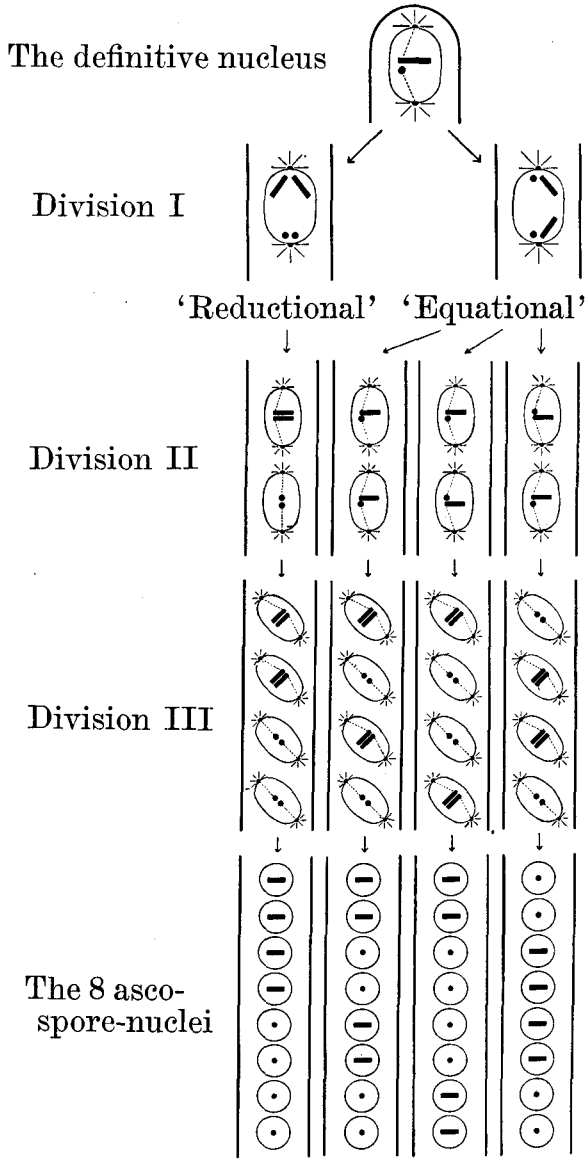
(ii) *Significance of the heteromorphic homologous chromosomes.* It would be rather premature to conclude that the heteromorphic pair of chromosomes found in the present material represents sex chromosomes, when the breeding tests to substantiate it fully are lacking, but it will not be denied that their behavior at nuclear fusion in the ascogenous hyphae and at meiosis strongly suggest that possibility.

All the available data from genetic works on the Ascomycetes point to the fact that heterothallism is governed by one-gene difference. The most instructive instance will be *Neurospora tetrasperma* which was investigated genetically by DODGE ('27, '29) and cytologically by COLSON ('34). In this fungus, the asci usually contain four large, initially binucleate spores. The mycelia raised from them are homothallic. Occasionally two small spores (initially uninucleate) take the place of one large spore. The mycelia produced from these small spores are heterothallic. Cytologically it was clearly demonstrated that the first division in the ascus is reductional and the spindles are formed in an oblique position at the second division, so that two non-sister nuclei become included in each normal spore. When these two nuclei are separated, small spores are produced. Thus it is clear that *N. tetrasperma* is homothallic only because of the inclusion of two different nuclei in each normal spore, and occasional occurrence of heterothallic spores is the result of segregation of the genes for sexuality at meiosis. The possibility of post-reduction of the sex genes has been also considered by DODGE and COLSON and in reality LINDEGREN ('32) inferred its occurrence in *N. tetrasperma, N. sitophila* and *N. crassa.* In the last-named fungus, he found 85% 'pre-reduction' and 15% 'post-reduction'. It must be then emphasized that in *Neurospora* we are dealing with only one pair of chromosomes for sexuality which divides either reductionally or less frequently equationally in the first division, although COLSON did

not observe any heteromorphic pair.

The present study is the first, the writers believe, to have demonstrated the existence of the hetero-chromosomes in fungi. If it be taken for granted that these are really sex chromosomes, they would have to be observed more frequently in heterothallic fungi, as in higher plants which are sexually differentiated. For this failure of their recognition, several possibilities may be considered: (1) that in these small objects it is seriously difficult to identify chromosome individuals morphologically; poor fixation would bring us nothing; (2) that since the mode of the first division, reductional or equational, may be probably subjected to environmental and genetic factors, one may meet with cases which are characterized by more frequent occurrence of the equational first division of heteromorphic pair than in the present case; the observation on these figures would readily lead one to take them as normal bivalents, and (3) that in species where the double fusion takes place, the recognition of their separation would be only possible at the third division, the nuclei at which stage are usually too small to permit the identification of them. In this connection, a figure drawn by MAIRE in 1905 and reproduced here in Plate X, Fig. 27, representing early anaphase stage of the first meiotic mitosis in *Peziza* (*Galactinia*) *succosa* is of interest, as it clearly shows a large rod-shaped chromosome in addition to seven eight small spherical ones, just similar to the writers' figures, though he considered it as a bivalent. Again BROWN ('11) notes at meiosis of *Patella* (*Lachnea*) *scutellata* the presence of one or two large chromosomes in addition to five small bivalents, though he mentions the former as 'granules' bearing a striking likeness to chromosomes. Critical cytological work on these materials and in other species of the Discomycetes is therefore desirable in order to ascertain the presence of such a heteromorphic pair. It may be added here in this connection that a similar pair has been actually demonstrated by the present writers in another species, *Helvella ephippiodies*, as clearly indicated by Fig. 59 of Plate XII, a microphoto of its metaphasic plate of the first division. The full description of its meiosis will be given in another paper.

The work on *Neurospora tetrasperma* carried out by DODGE and COLSON seems to be of significance in consideration of the nature of homothallism in the Ascomycetes. Its homothallic nature is apparently different from that of other eight-spored fungi, but both types can be brought into the same line by assuming that the usual homothallic species are of polyploid nature, at least as to the sex chromosomes, so that each of the eight spores come to contain both components of them. Whether this conjecture is



Text-fig. 1. Diagrammatic representation of the modes of reduction and the arrangements of resulting spores in the ascus.

correct or not, must depend upon further critical experimental and cytological works.

If the suggestion is correct that the heteromorphic pair as described in the present paper may represent real sex chromosomes, the arrangements of + and − spores in the ascus would be as shown in the following schemes (Text-fig. 1). It is natural that the arrangement in the row is determined by two factors: (i) the mode of reduction and (ii) the direction of the spindle axes of the three divisions. In the present case, the first division is usually reductional, with the exception of rare occurrence of the second division of a reductional nature, and the spindle axes of the first and the second division are in a longitudinal direction with regard to the length of the ascus, and that of the third division is oblique or transverse. Further studies must be undertaken in order to ascertain whether the present interpretation is really supported by fact or not.

It may be noted here that the occurrence of such equational splitting of the sex chromosomes at meiosis has been recently demonstrated cytologically by KOLLER & DARLINGTON ('34) in *Rattus* and interpreted on the basis of the chiasmatypy hypothesis. In this connection, a symmetrical form of metaphase association in the heteromorphic pair as represented in Fig. 50 of Plate XII will be of interest in that it suggests, as these authors interprete, the structure following crossing-over at chiasmata in both arms, the spindle attachments of the homologues being within the pairing segment.

Summary

The present cytological data for *Peziza subumbrina* may be profitably summarized as follows.

(1) The definitive nucleus of the ascus is diploid with 4 gemini, one of which is a heteromorphic pair composed of a large rod-like chromosome and a small spherical one.

(2) Usually the heteromorphic pair undergoes the first division of a reductional nature; very rarely it divides in an equational manner.

(3) No evidence of brachymeiosis is obtained in the present material. The haploid number of chromosomes is thus 4.

(4) The possibility of the heteromorphic pair representing sex chromosomes is considered.

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Explanation of Plates

All figures in Plates IX and XI, and Figs. 16, 17, 19, 20, 26, 29, 32–34 in Plate X were drawn by the junior writer, GONDÔ, with a Zeiss 2 mm. N.A.1.3 obj. and a Leitz oc. ×15 or ×25. The other figures in Plate X excluding Fig. 27, were drawn by the senior writer, MATSUURA, with a Zeiss 1.5 mm. N.A.1.3 obj. and a Zeiss comp. oc. 20. The photomicrographs in Plate XII were taken by the senior writer, with the aid of a Leitz photomicrographic apparatus, MA II, with a Zeiss 1.5 mm. N.A.1.3 obj. and Homal IV or comp. oc. ×30, Wratten filters B and E in combination, or B singly being interposed.

Plate IX

Figs. 1–15. ×3500

Fig. 1. Two nuclei in the ascogenous hyphae and the hook formation.
Fig. 2. Division of the nuclei in the ascus hook; the nuclei are in telophase.
Fig. 3. Four-nucleate condition of the ascus hook; the two nuclei in the penultimate cell will fuse in the young ascus.
Fig. 4. Two nuclei in young ascus before fusion.
Fig. 5. Nuclear fusion in the ascus.
Fig. 6. Young ascus with fusion nucleus in which two nucleoli are seen.
Fig. 7. Matured ascus; the nucleus is in resting stage.
Fig. 8. Synizesis.
Fig. 9. Early diplotene stage, showing extrusion of chromatin bodies at the ends of the spiremes.
Figs. 10 and 11. A somewhat later stage; the spiremes lose their chromaticity to a certain extent. Note the connecting filament between the extruded bodies and the nucleus.
Fig. 12. Mid-diplotene stage.
Fig. 13. Late diplotene stage.
Figs. 14 and 15. Second contraction; two centrosomes and the spindles between them are visible in Fig. 15.

Plate X

Figs. 16, 17, 19, 20, 26, 29, 32–34. ×3500.
Figs. 18, 21–25, 28, 30, 31. ×3200.

In these figures, the heteromorphic pair of chromosomes is represented by aa', a standing for the long member, a' for the short one.

Fig. 16. Division of the two nuclei in young ascus; the nuclei are in anaphase.
Fig. 17. Prometaphase; 4 gemini are didstinguished.
Fig. 18. Polar view of metaphase.
Figs. 10–26. Side views of anaphase; the heteromorphic pair divides reductionally.
Fig. 27. Metaphase in *Peziza succosa*. After MAIRE.
Fig. 28. Side view of anaphase; the heteromorphic pair divides equationally.
Fig. 29. Side view of late anaphase; the heteromorphic pair divides reductionally and all the separated chromosomes show second splitting.
Fig. 30. End of anaphase with lagging of a_1 and a_2 following equational separation of the long chromosome of the heteromorphic pair.
Fig. 31. Metaphase of the second division; the lower nucleus contains the long chromosome.
Fig. 32. Metaphase in one nucleus of the second division.
Fig. 33. Metaphase in one nucleus of the third division; 4 chromosomes are distinguished, one of which is long.
Fig. 34. Polar view of one set of separated chromosomes at anaphase in one nucleus of the third division; all 4 chromosomes are short.

Plate XI

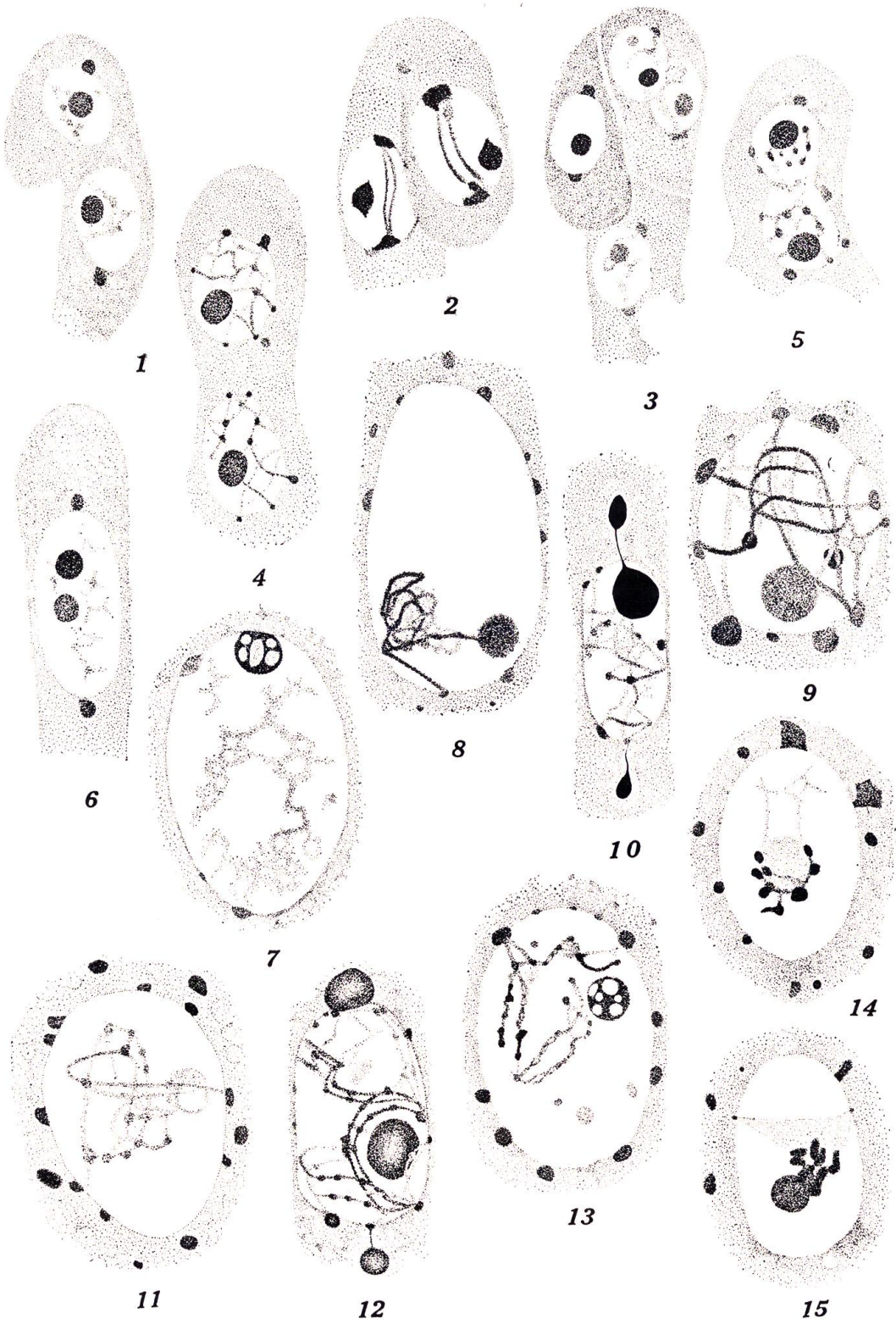
Figs. 35, 36, 41, 43. ×3500.
Figs. 37–40, 42. ×2000.

Fig. 35. Telophase of the first division; the nuclear membrane still persists.
Fig. 36. A later stage of the same; the nuclear membrane disappears and the astral radiation becomes more evident.
Fig. 37. Two nuclei in the ascus at metaphase of the second division.
Fig. 38. Four nuclei in the ascus at anaphase of the third division.
Fig. 39. Telophase of the same.
Fig. 40. Eight young spores, each of which contains a single nucleus.
Fig. 41. Prophase in one nucleus of the third division, showing synizetic contraction of chromatin.
Figs. 42 and 43. Anaphase in two daughter nuclei of divisions 3 and 2 respectively, showing a bundle of spindle fibers and its fragments in the nuclei.

Plate XII

Figs. 44–47, 49–56, 58, 59. ×1920.
Figs. 48 and 57. ×3840.

Fig. 44. Same as Fig. 17 in Plate X.
Fig. 45. Same as Fig. 30 in Plate X.
Fig. 46. Telophase of the first division, showing the direction of spindle formation.
Fig. 47. Telophase of the second division, showing the direction of spindle formation.
Fig. 48. Same as Fig. 20 in Plate X.
Figs. 49–54. Anaphase of the first division, showing the heteromorphic pair of chromosomes. Note the hetero-chromosomes lying off the plate.
Fig. 55. Early prometaphase of the first division; the heteromorphic pair develops earlier than the others which are still crowding around the nucleolus.
Fig. 56. Two nuclei at anaphase of the second division; in the upper nucleus the long chromosome is visible.
Fig. 57. Same as Fig. 32 in Plate X.
Fig. 58. Same as Fig. 34 in Plate X.
Fig. 59. Anaphase of the first meiotic division in *Helvella ephippioides*.



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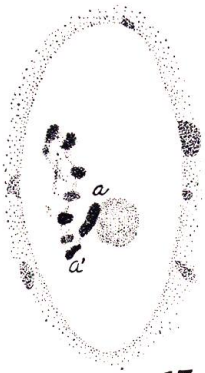
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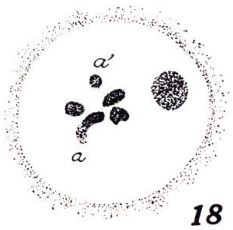
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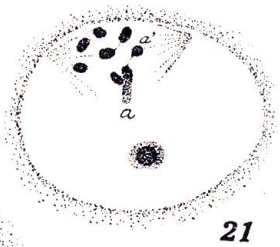
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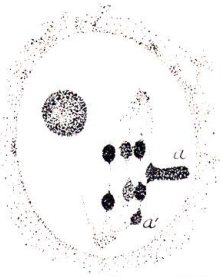
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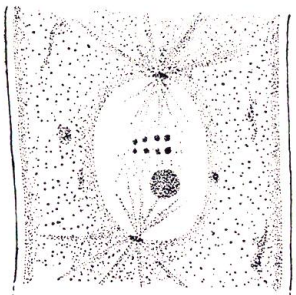

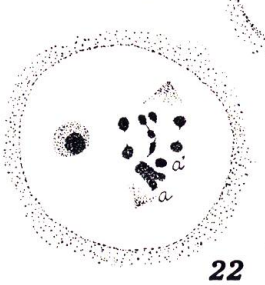
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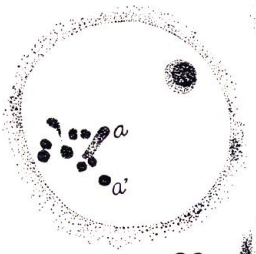
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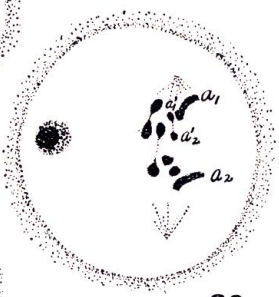
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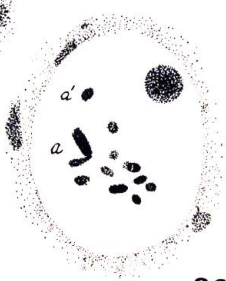
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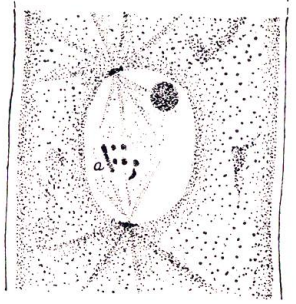
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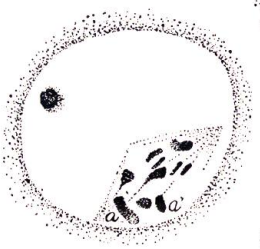
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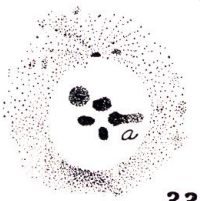
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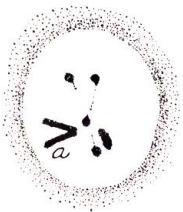
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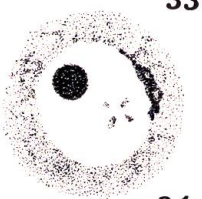
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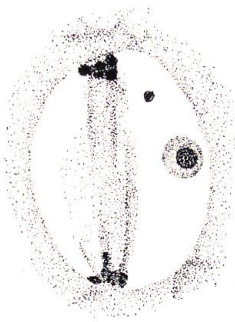

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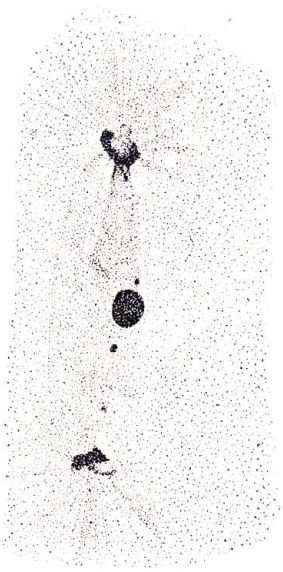

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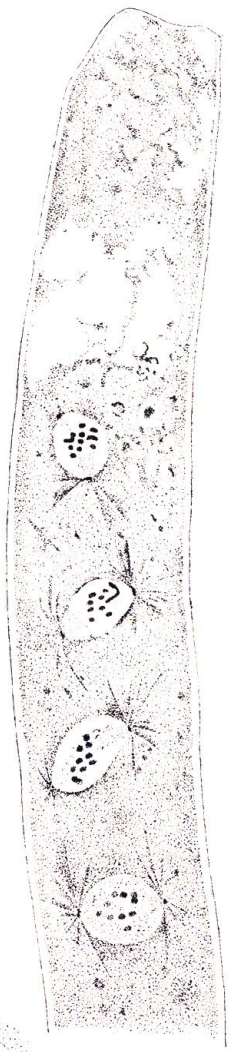

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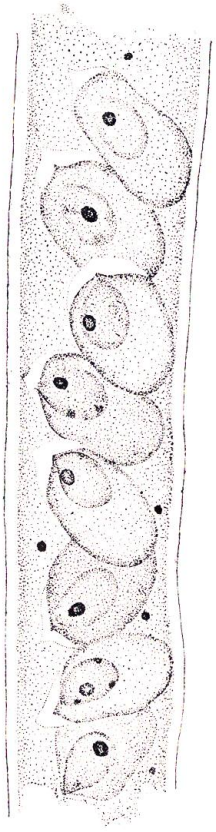

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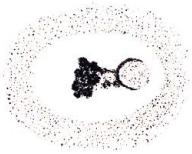

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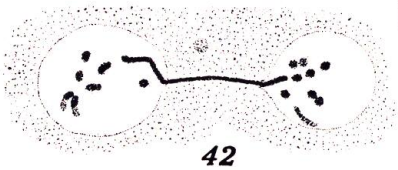

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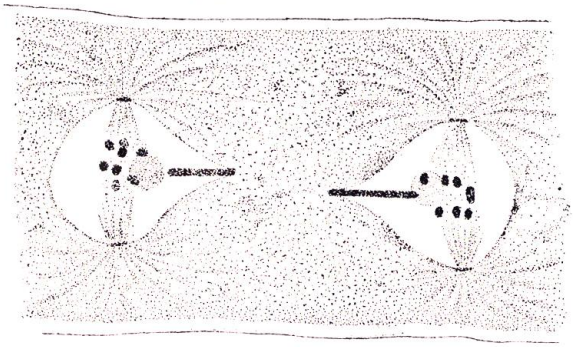

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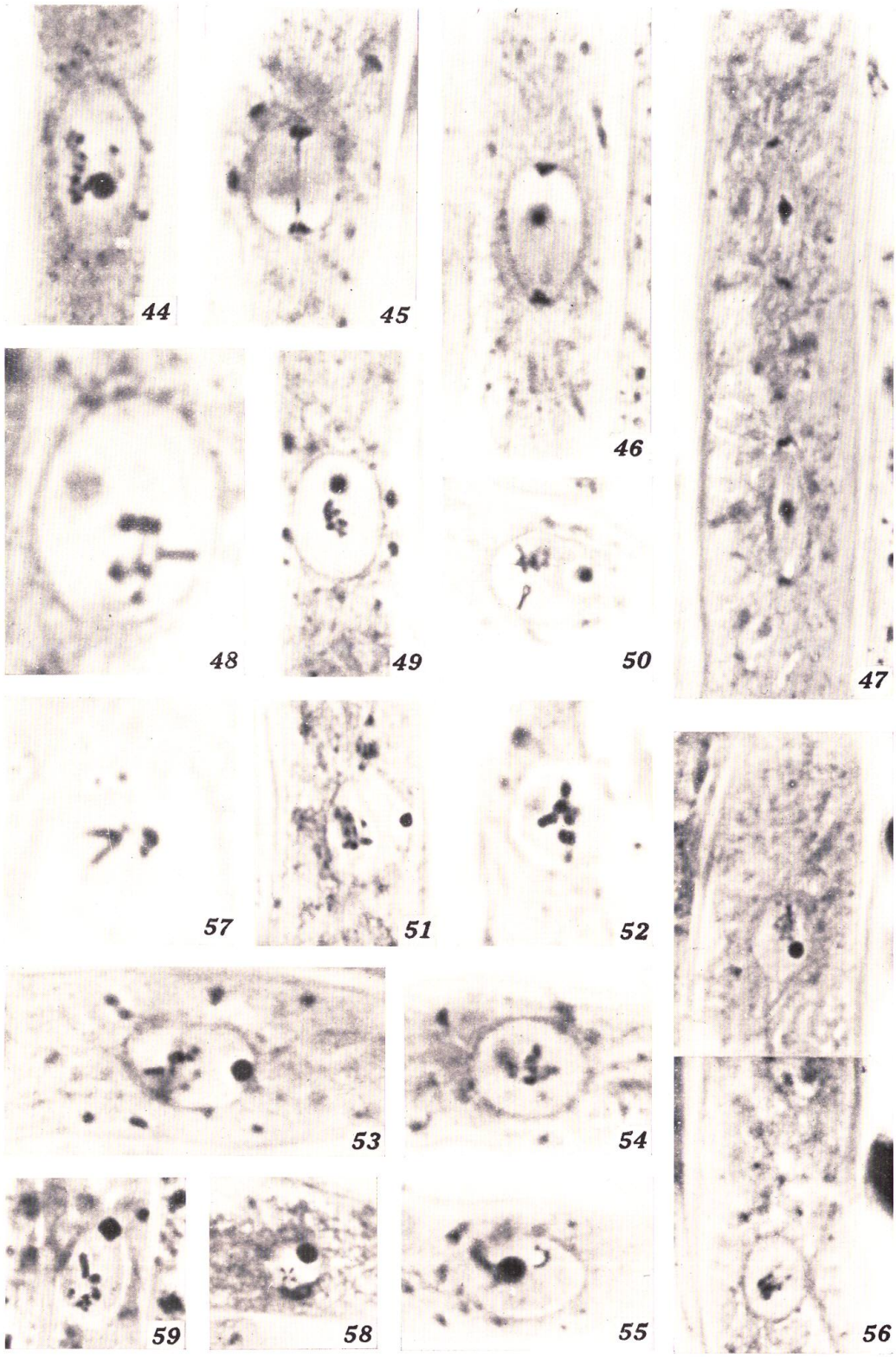

H. Matsuura photo.

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