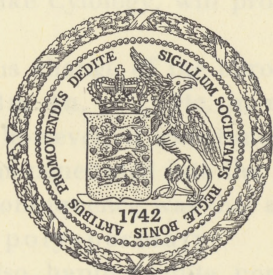


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THE SPONTANEOUS
FORMATION OF HAPLOID, POLYPLOID,
AND ANEUPLOID EMBRYOS
IN SOME ORCHIDS

BY

O. HAGERUP



KØBENHAVN

I KOMMISSION HOS EJNAR MUNKSGAARD

1947

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1. Introduction.

It is a fact that polyploid forms are of common occurrence in nature and play an important part both in the spontaneous formation of new species as well as in their ecological orientation. The problems of polyploidy therefore affect central biological questions, and the study of these is of decisive importance.

We know very little, however, of the ways in which the species change their degree of polyploidy in nature, and independently of man's intervention.

It has come to light, however, that the chromosome number of a species can undergo even very considerable changes during the fertilisation, which may differ remarkably in the different ovula found in the same ovarium. In the collective species *Orchis maculatus* L., embryos thus develop either n , $2n$, $3n$, $4n$, or $6n$ (and perhaps also other numbers of) chromosomes (HAGERUP, 1944).

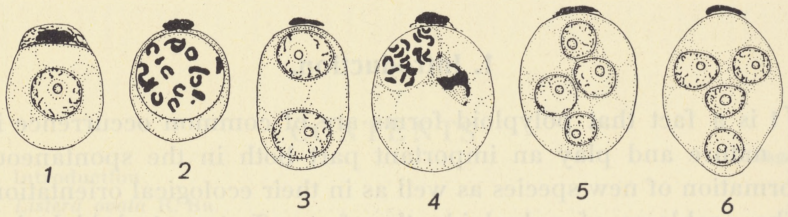
It would seem therefore that Embryology, one of the neglected children of Botany, like Cytology, will prove to be an important auxiliary science.

Of the spontaneous change of the chromosome number of a species from $2n$ to $3n$, $4n$, . . . etc. in nature little is known. In *Orchis maculatus*, however, it sometimes happened that more than one male nucleus penetrated into the female nucleus during fertilisation and the consequence was that a polyploid embryo was produced by polyspermaty.

Further it will also happen quite normally that some few per cent. (e. g. 5—10) of the ovula in an ovarium never receive any male nucleus but nevertheless continue their development and form a parthenogenetic embryo which is haploid, with the chromosome number n .

Since the various embryos receive differing numbers of genes from the father, they become genetically different (new species), and also react in different ways to the environment.

To throw light upon conditions of such decisive importance as the abovementioned it is necessary to use as large a material as possible. In addition to *Orchis*, therefore, *Epipactis latifolia*



Figs. 1—6. *Listera ovata*. First stages in development of embryo sac. $\times 550$.

was studied (HAGERUP, 1945), with the result that this species, too, forms haploid embryos by "facultative parthenogenesis".

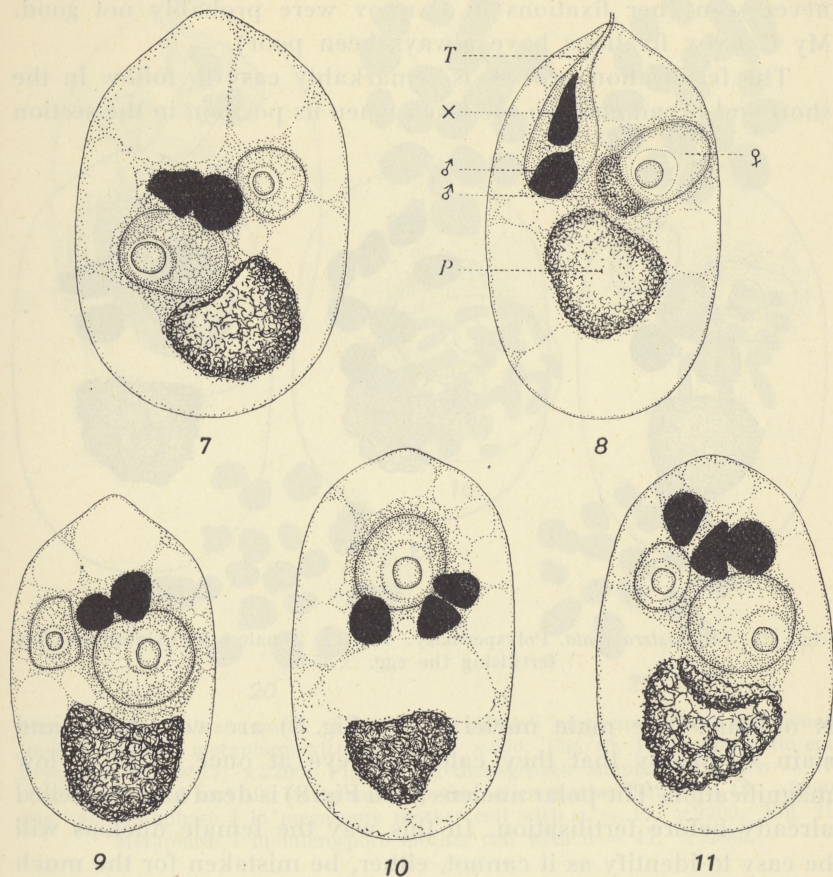
Below it will, moreover, be shown that the same is the case with several other orchids, which also develop haploid embryos without being fertilised.

These species were all gathered in nature in Denmark. They were fixed on the spot according to LEWITSKY and stained with FEULGEN, with "lichtgrün" as a contrast. This technique yields unique results, and it should be noted that the ovaria of the orchids (more particularly *Listera* and *Neottia*) are ideal pedagogic objects, because the most delicate details, otherwise so difficult to see in most other plants, are easy to study here.

2. *Listera ovata* R. BR.

Several investigations, recent too, are available both on the haploid and the diploid chromosome number (TUSCHNJAKOVA, 1929 and RICHARDSON, 1933). Most commonly n is 17, but often n may also be 16, 18 or 19. TUSCHNJAKOVA has given an excellent description of how these interesting aneuploid numbers arise by irregularities of meiosis (non-disjunction).

So it will here suffice to append some figures showing that plants from Møn and the vicinity of Copenhagen have $n = 17$



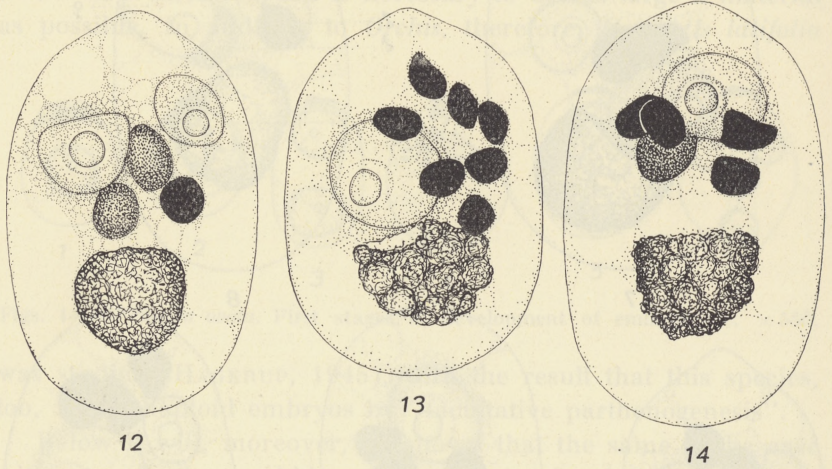
Figs. 7—11. *Listera ovata*. Fertilisation. *T*, pollen tube; ♂, male nucleus; ♀, female nucleus; *P*, polar nucleus; *X*, tube nucleus. The two male nuclei often adhere to each other. $\times 550$.

(Figs. 20 and 21), whereas n is 18 (Figs. 18, 19) in plants from calcareous soil in central Sealand (Alindelille).

The development of the remarkable embryo sac has also been well investigated by TUSCHNJAKOVA, so I will here merely figure (Figs. 1—6) the first stages of the development. No antipodals are formed, and the mature embryo sac merely seems to contain 4 nuclei; the lower of these (the polar nucleus) may, however, divide, so that it consists of two or more partly fused nuclei. TUSCHNJAKOVA even states that she has found as many as 8 nuclei in embryo sacs of plants from Russia, but that I have

never seen (her fixations in CARNOY were probably not good. My CARNOY fixations have always been poor).

The fertilisation process is remarkably easy to follow in the short and broad embryo sac, even when its position in the section

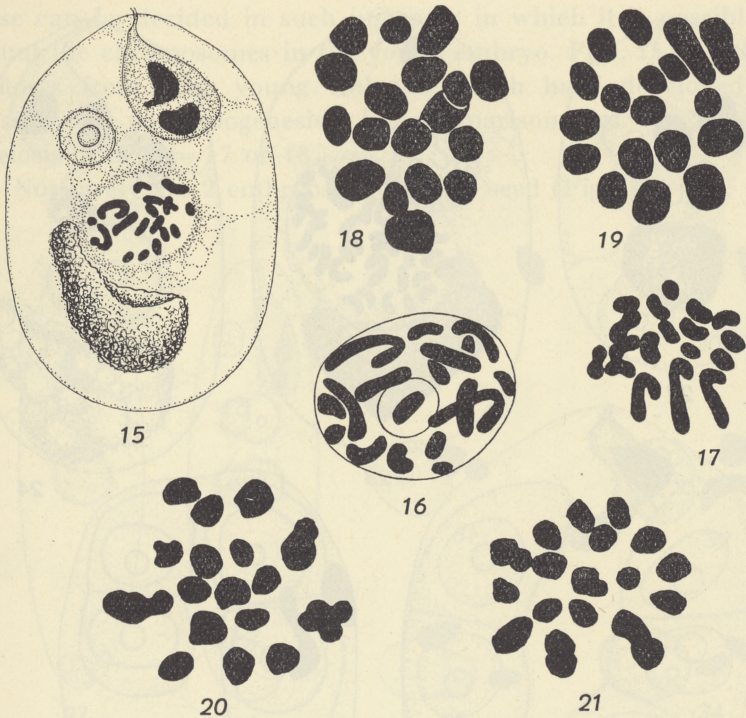


Figs. 12—14. *Listera ovata*. Polyspermaty. Fig. 12. 2 male nuclei simultaneously fertilising the egg. $\times 550$.

is oblique. The male nuclei (σ in Fig. 8) are very large and stain so deeply that they catch the eye at once, even at low magnification. The polar nucleus (*P* in Fig. 8) is dead and shrivelled already before fertilisation. In this way the female nucleus will be easy to identify as it cannot, either, be mistaken for the much smaller synergids. It is therefore possible in a very short time to follow a large number of fertilisation stages and obtain a reliable numerical material.

When the tip of the pollen tube enters the embryo sac, it kills one synergid and swells considerably. Most frequently all the 3 nuclei of the pollen tube enter the embryo sac. The 2 male nuclei frequently pass together to the female nucleus; and often they even adhere (Figs. 7, 11) so closely to each other that they both fuse with the female nucleus simultaneously (Fig. 12).

The polar nucleus, which is dead, is never fertilised, and no endosperm forms. Hence a male nucleus will as a rule be left over, and this will be found lying next to the young embryo (Figs. 25, 26).

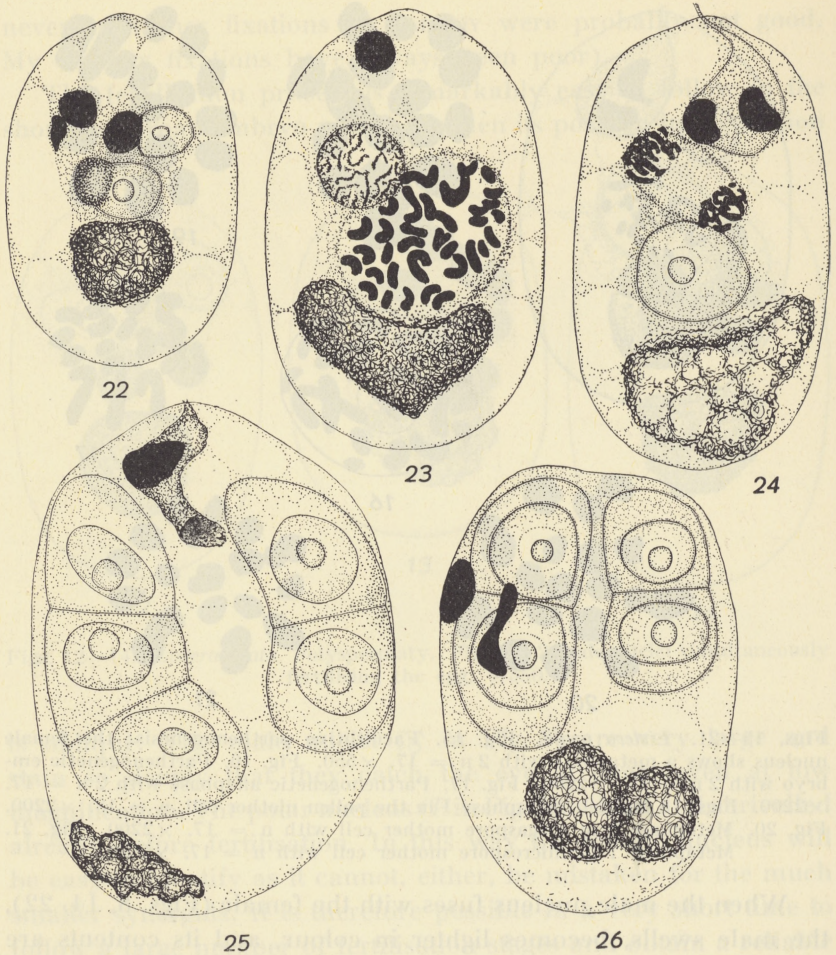


Figs. 15—21. *Listera ovata*. Fig. 15. Facultative parthenogenesis: the female nucleus shows a metaphase with $2n = 17$. $\times 550$. Fig. 16. Parthenogenetic embryo with $2n = 17$. $\times 2200$. Fig. 17. Parthenogenetic anaphase with $2n = 17$. $\times 2200$. Figs. 18 and 19. Metaphase I in the pollen mother cell, $n = 18$. $\times 2200$. Fig. 20. Metaphase I in megaspore mother cell with $n = 17$. $\times 2200$. Fig. 21. Metaphase I in microspore mother cell with $n = 17$. $\times 2200$.

When the male nucleus fuses with the female (Figs. 8, 14, 22), the male swells, becomes lighter in colour, and its contents are resolved into fine grains.

It is a common occurrence that an embryo sac receives more than one pollen tube, and more than 2—3 male nuclei will then lie next to the female nucleus (Figs. 13, 14). In one case (Fig. 12) it was even seen that a female nucleus was fertilised by 2 male nuclei simultaneously. That this may lead to the formation of triploid embryos was found in *Orchis maculatus*, but not in *Listera* whose chromosome number is relatively difficult to determine.

The distribution of the numerous pollen tubes among the ovula does not merely go wrong in that one ovulum may receive

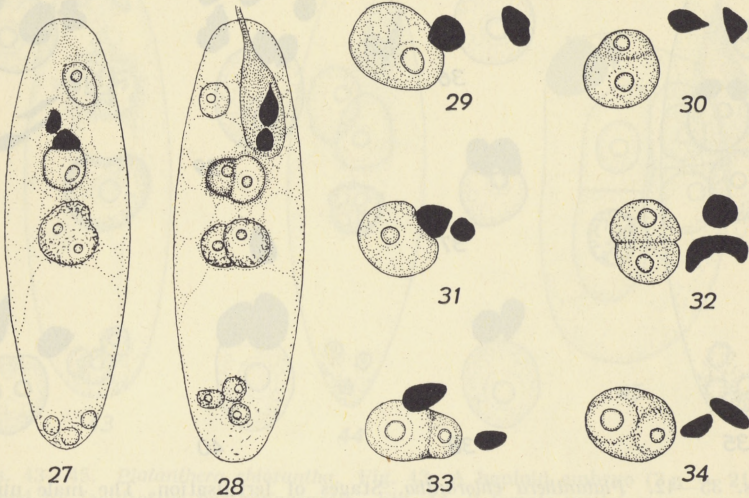


Figs. 22—26. *Listera ovata*. Development of twins. Fig. 22. Both egg and synergid seem to be fertilised. Fig. 23. The female nucleus forms a diploid embryo ($2n = 34$), and the synergid also has begun to develop an embryo. Fig. 24. The egg is undivided but the synergid has begun to form an embryo. Figs. 25—26. Few-celled twins. $\times 550$.

more than one pollen tube. It may also sometimes happen (in about 5 p.c. of the ovula) that an ovulum does not receive any pollen tube at all, or the latter may come too late (Fig. 15). Such an unfertilised ovulum does not die, however, but the female nucleus begins to divide and develop a parthenogenetic embryo which will then be haploid. That this is actually the

case can be decided in such instances in which it is possible to count the chromosomes in the young embryo. Figs. 15—17 show mitoses from such young embryos which have developed by "facultative parthenogenesis". For comparison Figs. 18—21 show meiosis with $n = 17$ or 18.

Not rarely are 2 embryos found in a seed (Figs. 23—26). One



Figs. 27—34. *Platanthera chlorantha*. Stages of fertilisation. The male nuclei keep apart. Figs. 27—28 $\times 700$. Figs. 29—34 $\times 1500$.

of these has then been formed of a synergid, which in some instances (Fig. 22) seems to be fertilised, hence it must be diploid. If a synergid develops without fertilisation, the result is a haploid embryo.

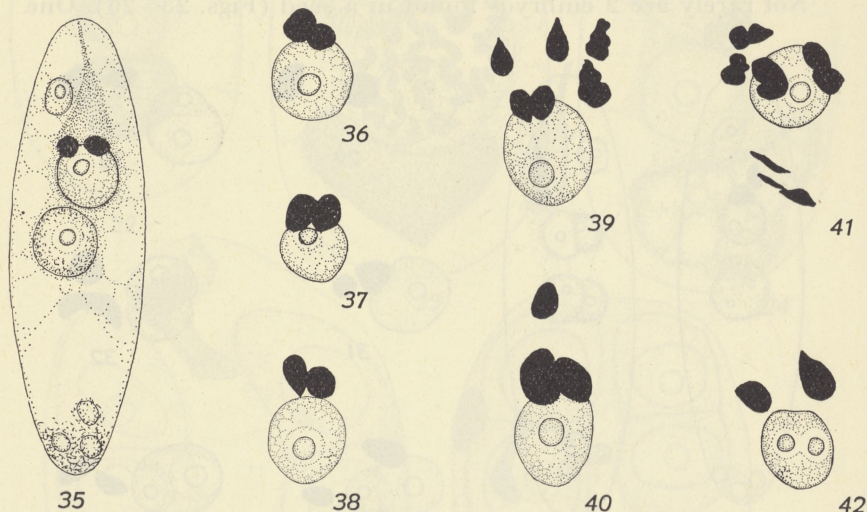
3. *Platanthera chlorantha* (CUSTER) Rchb.

The structure and development of the embryo sac have been closely studied by AFZELIUS (1922), so a reference to his work will suffice.

The tip of the pollen tube sometimes contains only the 2 male nuclei. One of the synergids is killed by the pollen tube, which swells considerably at the tip. AFZELIUS states (p. 377) that one of the male nuclei fuses with the polar nucleus. This I have never seen, though I have examined many thousands of ovula

and seen many hundred fertilisation stages. Moreover the polar nucleus is dead when the egg is fertilised, and I have never found any endosperm. The 3 small antipodals too are dead in the ripe embryo sac and soon perish.

The fusion of the two sexual nuclei is remarkably easily



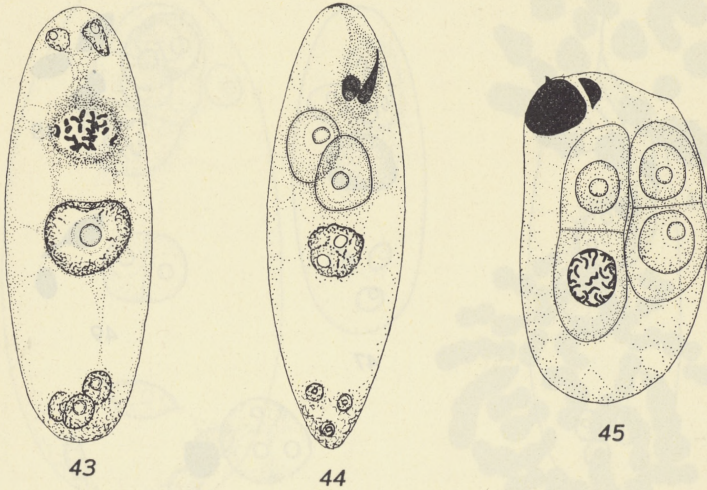
Figs. 35—42. *Platanthera chlorantha*. Stages of fertilisation. The male nuclei adhere firmly to each other (superfertilisation?). Fig. 35. One synergid has developed as a sexual cell (cause of twins).

studied because this process is very slow in *Platanthera* (Figs. 27—34). In some instances the 2 male nuclei do not pass together to the female which is then only fertilised by one nucleus (Figs. 27—34), and the embryo will then be diploid ($2n = 42$).

Nor is it rare that the 2 male nuclei adhere so closely to each other (Figs. 36—38) that they must both enter the egg simultaneously. In addition it also often happens that an embryo sac receives more than one pollen tube, the male nuclei of which then place themselves in the immediate vicinity of the egg, which means that there is a chance of polyploid embryos being formed (Figs. 39—41).

As in the above-mentioned species, it may also happen in *Platanthera* that an ovulum which does not receive any pollen tube develops a parthenogenetic embryo, which is haploid (Fig. 43) with $n = 21$.

Fig. 44 shows an embryo sac, one synergid of which is developed as a female nucleus. If the latter is not fertilised, a haploid embryo will arise in this way too ($2n = 21$, Fig. 45); it lies beside the normal diploid embryo, but may often be distinguished from the latter by a difference in the size of the nuclei.



Figs. 43—45. *Platanthera chlorantha*. Fig. 43. A haploid embryo ($2n = 21$) is formed by facultative parthenogenesis. Fig. 44. 2 female nuclei may cause the formation of twins. (Fig. 45) $\times 800$.

4. *Orchis strictifolius* OPIZ. (= *O. incarnatus* L.).

The embryo sac contains 8 nuclei (Fig. 46) of which both the antipodals and the polar nucleus are dead when fertilisation takes place, so that no endosperm develops.

Fig. 47 shows that a parthenogenetic embryo has developed (with $2n = 20$) in a embryo sac which has not received any pollen tube.

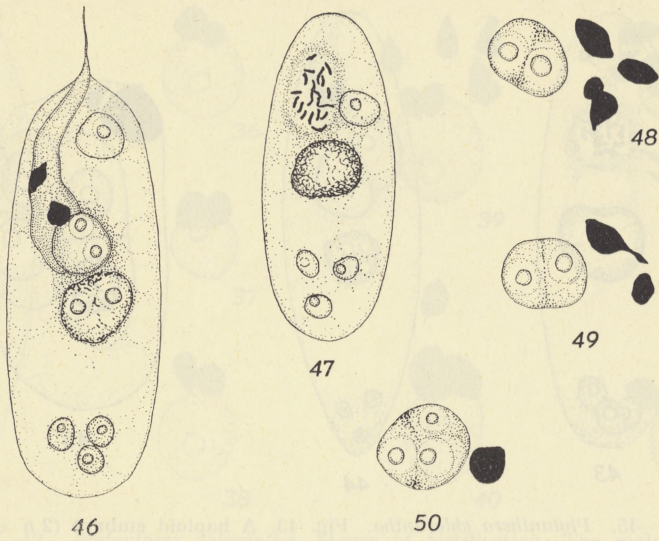
In an embryological respect the species shows similar conditions to *O. maculatus*.

5. *Cephalanthera longifolia* (L.).

Owing to their rarity the *Cephalanthera* species have not been subjected to embryological investigations. AFZELIUS (1943) found

that *C. longifolia* has $n = 16$ in Sweden, and I have found the same number in plants from S. Sealand (Oreby Forest, Fig. 52).

The development and structure of the embryo sac entirely resemble that of *Epipactis*; the ripe embryo sac contains 8 nuclei. In contrast with most other orchids the polar nucleus is really



Figs. 46—50. *Orchis strictifolius (incarnatus)*. Stages of fertilisation. Fig. 47. Development of a haploid parthenogenetic embryo with $2n = 20$. $\times 800$.

fertilised and develops an endosperm consisting of quite few nuclei that die quickly, so that the ripe seed does not contain an endosperm.

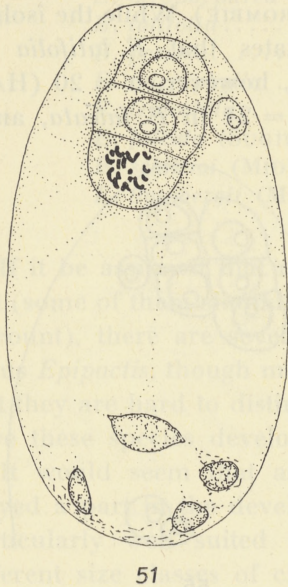
In this species too a few parthenogenetic haploid embryos develop ($2n = 16$, Fig. 51) when an ovulum is not fertilised.

6. *Cephalanthera Damasonium* (MILL.).

Of this magnificent plant abundant material was gathered in Møn, where the fertilisation stages occur at the close of July.

BARBER found that $n = 18$, but the plants from Denmark showed $n = 16$ and $2n = 32$ (Fig. 53). It is possible therefore that the species contains aneuploid forms.

The structure of the embryo sac is as in *C. longifolia* and *Epipactis*; here too there are 8 nuclei in the embryo sac. The polar nucleus is also fertilised and develops a paucinuclear endosperm (Fig. 54) which is soon absorbed again.



51



52



53

Figs. 51—53. *Cephalanthera*. Fig. 51. *C. longifolia*. Development of a parthenogenetic haploid embryo with $2n = 16$. $\times 500$. Fig. 52. *C. longifolia*. Metaphase I in megaspore mother cell with $n = 16$. $\times 2400$. Fig. 53. *C. Damasonium*. Metaphase I in young embryo with $2n = 32$. $\times 2400$.

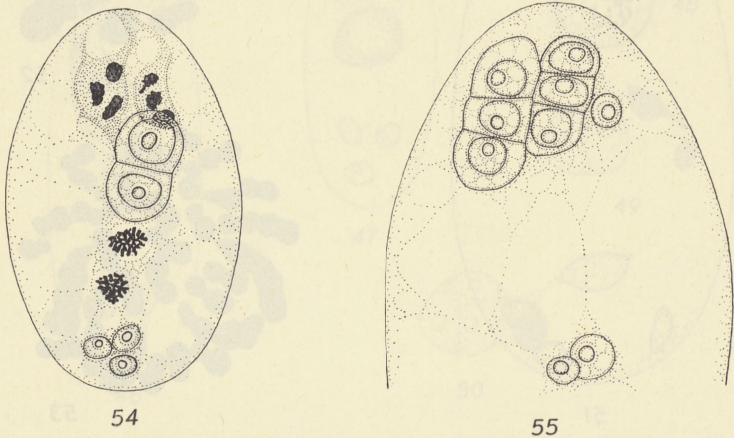
Not rarely more than one pollen tube (Fig. 54) enters the embryo sac and superfertilisation is then possible.

It is also a fairly common occurrence that one of the synergids forms an embryo so that the seed will contain twin embryos (Fig. 55).

It is, however, of special interest that in this species too the female nucleus may develop without fertilisation, if for instance the pollen tube should "come too late", as shown in Fig. 56. The consequences of such a "facultative parthenogenesis" will then again be that a parthenogenetic embryo will develop, with $2n = 16$.

7. Aneuploidy (*Epipactis* sp.).

The species grouped around the main species, *E. latifolia*, are interesting subjects of study because they are markedly polymorphous (NANNFELDT, ABERCROMBIE). While the isolated *E. palustris* has $n = 20$, BARBER states that *E. latifolia* has $n = 19$. In Denmark this species has, however, $n = 20$ (HAGERUP, 1945). SUGIURA even found $2n = 24$ in *E. falcata*, and I



Figs. 54—55. *Cephalanthera Damasonium*. Fig. 54. 2 pollen tubes in embryo sac. Incipient formation of endosperm. Fig. 55. Twins. $\times 500$.

found $2n = 36$ in *E. leptochila* (Fig. 58). It would seem therefore that the genus presents an interesting subject for studies on the bearing of aneuploidy on the formation of species. Hence the genus *Epipactis* should be made the subject of thorough research in as many countries as possible.

NANNFELDT has made a thorough and excellent revision of the Scandinavian *Epipactis* species. I have been able to fix most of these forms in nature. *E. persica* HAUSSKN. was gathered in Møn, and Chief Veterinarian G. HARBOU gave me a living specimen of *E. leptochila* (GODF.) from S. Funen (Fig. 58). My special thanks are, however, due to Dr. C. HEUSSER who after great difficulties managed to fix the smallest species, *E. microphylla* (EHRH.), in Switzerland (Fig. 57).

Examination of the root tips showed that $2n = 40$ in *E.*

microphylla (Fig. 57). Altogether the following chromosome numbers have been recorded within *Epipactis*:

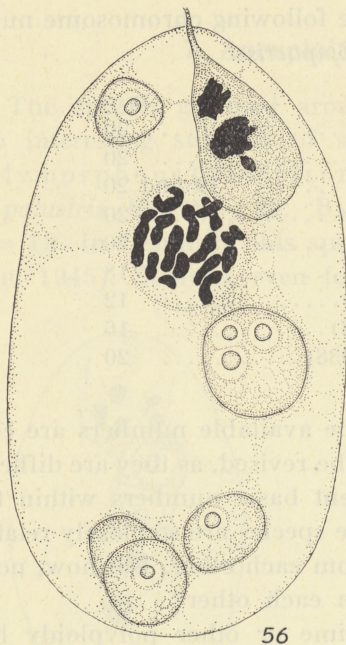
	n =
<i>E. palustris</i>	20
<i>E. atropurpurea</i>	20
<i>E. latifolia</i>	19 and 20
<i>E. persica</i>	20
<i>E. microphylla</i>	20
<i>E. leptochila</i>	18
<i>E. falcata</i> (SUGIURA)	12
<i>E. schizuoi</i> (MIDUNO, 1938)	16
<i>E. thunbergii</i> (MIDUNO, 1938)	20

If it be assumed that most of the available numbers are correct (some of them should perhaps be revised, as they are difficult to count), there are several different basic numbers within the genus *Epipactis*, though many of the species are so closely related that they are hard to distinguish from each other. But how, now, have these species developed from each other?

It would seem that at some time or other polyploidy has played a part in the development of some of the species; for in particularly well suited stages of division with $2n = 40$ the different size classes of chromosomes are present in a number of 4, and often lying near each other, as indeed is frequently the case with polyploids.

But the phenomenon of polyploidy does not give the whole explanation of how species with such basic numbers as 12, 16, and 18 can have developed or been derived from each other or perhaps from 20. These numbers are perhaps aneuploid? To throw light on this question numerous pollen tetrads were examined, and it then turned out that in every single microsporangium some few per cent. of the tetrads were very regularly found which, in addition to the normal 4 microspores, also contained one or more extra microspores (Figs. 59, 60, 70). The accessory pollen grain is remarkably small, not only in volume, but the nucleus evidently contains much less chromatin than normal pollen grains.

Similar dwarfish pollen is known from many other orchids and in many other plant families, and their development has been thoroughly investigated, e. g. by AFZELIUS, FERNANDES and TUSCH-



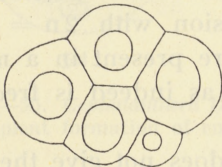
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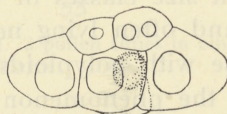
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Figs. 56. *Cephalanthera Damasonium*. Facultative parthenogenesis. The egg forms a haploid embryo with $2n = 16$. $\times 1100$. Fig. 57. *Epipactis microphylla* (Switzerland). Metaphase from root tip with $2n = 40$. $\times 2400$. Fig. 58. *Epipactis leptochila*. Metaphase from root tip with $2n = 36$. $\times 2400$. Figs. 59—60. *Epipactis persica*. Pollen tetrads with extra dwarf pollen. $\times 1000$. See also text.

NJAKOVA, who found that one or several chromosomes may be entirely ejected in meiosis and form an independent small pollen grain. In other instances two sister nuclei do not receive the same number of chromosomes, one or several gemini migrating, divided or undivided, to one single nucleus, which thus receives more chromosomes than the sister nucleus; the latter has then a lower and aneuploid number (non-disjunction).

In our Danish *Epipactis* species there are, almost in every

section through a pollen sac, certain microspores from which arise gametes with a deviating chromosome number. Already prior to the prophase stages in meiosis, some few nuclei may thus be found, scattered among the normal pollen mother cells, which are predestined to form tetraploid pollen grains (Figs. 65, 66). These nuclei have a particularly dense content and are therefore also comparatively highly susceptible to staining. In successful staining it may even be possible to get the normal nuclei stained green, and between these the tetraploid mother nuclei will then appear, stained a vivid scarlet and therefore especially conspicuous.

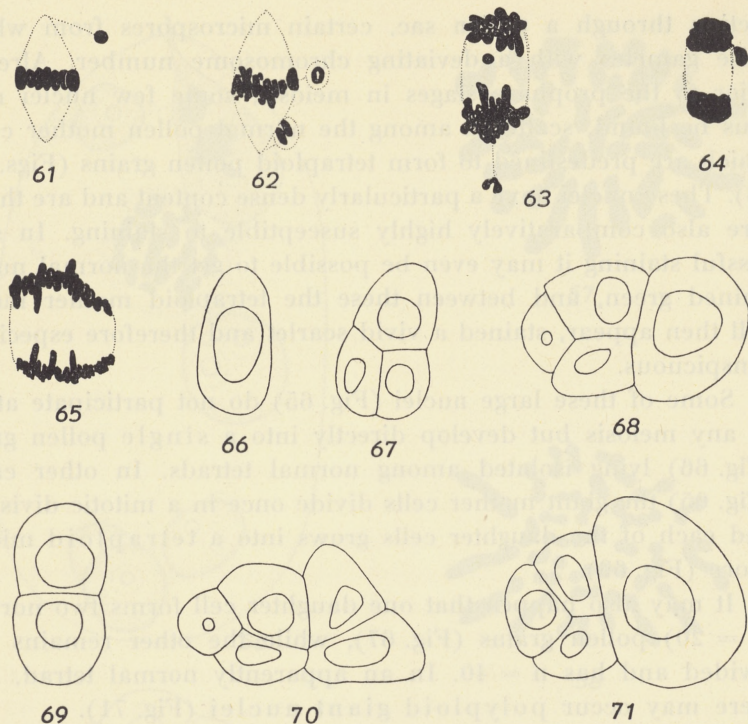
Some of these large nuclei (Fig. 65) do not participate at all in any meiosis but develop directly into a single pollen grain (Fig. 66) lying isolated among normal tetrads. In other cases (Fig. 65) the giant mother cells divide once in a mitotic division, and each of the daughter cells grows into a tetraploid microspore (Fig. 69).

It may also happen that one daughter cell forms two normal ($n = 20$) pollen grains (Fig. 67), while the other remains undivided and has $n = 40$. In an apparently normal tetrad, too, there may occur polyploid giant nuclei (Fig. 71).

If a polyploid female gamete is fertilised by a similar male gamete, a polyploid embryo may be formed. Polyploid *Epipactis* plants should be looked for in nature where giant plants are sometimes met with.

8. The Formation of New Species.

Thus *Epipactis* seems to be a standard example of how gametes with a deviating chromosome number may develop spontaneously in nature. And it is important to note that, though the species do not seem to be direct hybrids, yet there is normally distinct non-disjunction in the formation of a certain small number of pollen grains in each pollen sac. This affords a chance for the development of individuals with aneuploid chromosome numbers; such individuals must also have new genetic characters, and so they must also have the chance of becoming founders of new species. And as a matter



Figs. 61—71. *Epipactis latifolia*. Non-disjunction. During meiosis one or more uni- or bi-valent chromosomes are ejected (Figs. 61—64), and microspores are formed with different aneuploid numbers and nuclei of different sizes. The largest nuclei are polyploid (Figs. 65, 66, and 69). $\times 1000$. See also the text.

of fact, a number of such microspecies with deviating aneuploid numbers occur in nature. (But it must be borne in mind that there also occur microspecies with the same chromosome number, which have not developed in this way.)

The genus *Orchis* (HAGERUP) affords a particularly fine example of what an important part the fluctuations in the degree of polyploidy in nature may play for the spontaneous formation of species. In many other orchids too haploid embryos may develop by "facultative parthenogenesis". The higher degrees of polyploidy may also develop either by (1) "superfertilisation" or (2) by the normal formation of single polyploid pollen grains and gametes.

Quite schematically these processes may be expressed numerically by the fact that a chromosome number (e. g. $n = 20$) can

be multiplied (or divided) by e. g. 2, 3, 4, etc. or it may be subjected to a subtraction or an addition so that it is changed from $n = 20$ to $n = 20 \pm 1 \pm 2$ etc. Or a given number may be subjected to more than one of these processes.

That the above-mentioned conditions also occur perhaps in most other plants is illustrated by the fact that e. g. *Pyrolaceae* (HAGERUP) has the basic number 8. But in addition the family also contains the polyploid number 3×8 as well as the aneuploid number $3 \times 8 \div 1$ and even $(3 \times 8 \div 1) \times 2$. Others again of the family have $n = 13$ or 19, which numbers have presumably also arisen by non-disjunction. Within many other smaller or larger systematic units we find similar numerical conditions due to polyploidy and aneuploidy.

Most of the many chromosome numbers already known from other orchids seem to be aneuploid. It may be conjectured therefore that an essential part of the causes of the remarkably rich species formation in the orchids may partly be explained as phenomena connected with (1) aneuploidy and (2) fluctuations in the degree of polyploidy.

Such changes in the number of chromosomes (and the corresponding genetic composition) of the species thus take place spontaneously in nature. In this I see some of the chief causes of the spontaneous formation of species. How this species formation is then again dependent on, for instance, pollination and other external factors should be studied more closely.

My respectful thanks are due to the Carlsberg Foundation for the grants enabling me to pursue these studies, and to Professor C. A. JØRGENSEN who has given me much help with the difficult chromosome counts.

9. Summary.

1. The following 7 species were studied: *Listera ovata*, *Platanthera chlorantha*, *Orchis incarnatus*, *Cephalanthera longifolia*, *C. Damasonium*, *Epipactis microphylla*, and *E. leptochila*.
2. In nearly all of these species some few per cent. of the embryos were developed by "facultative parthenogenesis" and were thus haploid.

3. In some, polyploid embryos may perhaps also be formed, because (1) more than one male nucleus enters the egg (poly-spermaty) or (2) by polyploid gametes.
4. After irregularities in meiosis (non-disjunction) gametes are formed, with aneuploid chromosome numbers.
5. It is conjectured on general grounds that the circumstances associated with (1) aneuploidy and with the degree of fluctuation in (2) polyploidy are among the most important of the species-forming factors in nature. The dependence of these factors on external conditions should be subjected to close investigations.

Botanical Museum, Copenhagen 1947.

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