

# ADVENTITIOUS EMBRYONY IN *NIGRITELLA* (*ORCHIDACEAE*)

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**Abstract:** The embryology of diploid and polyploid *Nigritella* species is described. The development of the adventitious embryos of the polyploids in relation to the sexual embryos of the diploids has been given special consideration. Partial sexuality in the apomictic *N. nigra* has been proven for the first time by chromosome counts in proembryos after pollination of a tetraploid plant with pollen from a diploid species.

## INTRODUCTION

Adventitious embryony is known to occur in *Nigritella* since the work of AFZELIUS (1928, 1932) on *N. nigra* (L.) RCHB. f. subsp. *nigra* from Scandinavia. These papers remained for a long time the only source and are often cited. ASKER & JERLING (1992) have mentioned only these. In the meantime I have included the mode of reproduction as an important characteristic of the species, during a taxonomic investigation of this genus (TEPPNER & KLEIN 1985a,b, 1989, 1990, 1993, TEPPNER et al. 1994, TEPPNER 1991 a,b, ROSSI et al. 1987). The five diploid taxa, *N. carpatica* (ZAPAL.) TEPPNER, KLEIN et ZAGULSKIJ, *N. rhellicani* TEPPNER et KLEIN, *N. gabasiana* TEPPNER et KLEIN, *N. corneliana* (BEAUV.) GÖLZ et REINHARD, *N. lithopolitanica* RAVNIK, reproduce sexually whereas the five polyploid species, *N. nigra* (including *N. nigra* subsp. *nigra*, *N. nigra* subsp. *austriaca* TEPPNER et KLEIN and *N. nigra* subsp. *iberica* TEPPNER et KLEIN), *N. miniata* (CRANTZ) JANCH., *N. widderi* TEPPNER et KLEIN, *N. stiriaca* (K. RECH.) TEPPNER et KLEIN, *N. archiducis-joannis* TEPPNER et KLEIN, are apomictic and produce adventitious (nucellar) embryos.

## MATERIAL AND METHODS

Material other than that listed in TEPPNER & KLEIN 1985-1994: *Nigritella nigra* subsp. *austriaca* (2n=80): Lower Austria, Schneeberg, 22.VII.1986, leg. E. KLEIN & W. VÖTH; pollinated 1991 with pollen from *N. corneliana* (2n=40): Italy, Sestriere, 15.VII.1986, leg. H. TEPPNER & E. KLEIN. Gynoecia fixed 19.VI.1991. Grown in the Botanical Garden of the University of Graz.

The material for karyological investigations (gynoecia) was fixed in ethanol : chloroform : acetic acid (5:3:1), stained with a saturated solution of carmine in 45% acetic acid, and prepared by the usual squash method.

For the embryological and karyological studies of intact ovules, the same material and stain was used despite the disadvantage that the delimitation of cells is often not sufficiently discernible. The advantage gained is that the chromosomes remain clearly assessable in all



stages and only this fact permits the clarification of the whole mode of reproduction in the tenuinucellate ovules of *Nigritella*. The more elaborate method of microtome sectioning after the usual fixations for anatomy and embryology in formalin containing fixatives would have been too time consuming.

## Diploids

The basic chromosome number in *Nigritella* is  $x=20$ . Diploids therefore possess  $2n=40$  chromosomes.

## Embryology

The pollen grains are two-celled, the generative cell is clearly linked to the vegetative nucleus, forming the male germ unit (TEPPNER & KLEIN 1993: 183, 195, TEPPNER et al. 1994: 174). The generative cell divides in the tip of the pollen tube between the ovules (TEPPNER & KLEIN 1985: 157-158, TEPPNER et al. 1994: 178-179).

Megasporogenesis and megagametogenesis have been described more than once (TEPPNER & KLEIN 1985-1994). The lower cell of the tetrad forms the fertile embryo sac (ES). The ripe ES usually contains four nuclei: two synergids, an egg nucleus and a large pentaploid chalazal nucleus (four antipodal nuclei and the micropylar polar nucleus are fused). In the fertilization process only one sperm nucleus is needed for the fusion with the egg cell, because no endosperm is initiated (e.g. TEPPNER & KLEIN 1993: 185, 190-191).

The first embryonic development follows the Onagrad type (Fig. 1; TEPPNER 1991a,b, TEPPNER & KLEIN 1993: 186, 187, 192). The further development is in accordance with other *Orchideae* and which has been known for a long time (see e.g. WARD (1880) for *Gymnadenia* and VEYRET (1965: 16-19) for *Orchis*). Finally the suspensor grows far out of the outer integument and its tip is pressed against the placenta (TEPPNER & KLEIN 1993: 187). The egg cells and the zygotes are highly polarized cells and lie in the physiological gradient between the micropylar and the chalazal pole of the nucellus and the embryos develop very regularly. Until now no concrete observations of apomixis have been made in diploids.

## Twins

While examining a larger number of ovules sometimes a twin ovule (two ovules in the same outer integument, Fig. 2a; STRASBURGER 1878: Fig. 56 for *Gymnadenia conopsea*) can be found. Also twin nucelli (two nucelli in the same inner integument) are possible as a rare event. Twin embryos (two embryos in the same nucellus, Fig. 2b,c) are very rare and even in the observed cases mitoses were not seen, therefore we have no chromosome counts. To interpret twin ovules or twin nucelli (false polyembryony p.p., MAHESHWARI & SACHAR 1963: 265, 277-278), in which every part needs its own fertilization process, as apomixis (MRKVICKA (1994: 175); twin ovules in *N. rhellicani*) is incorrect.

## Spontaneous triploids

While investigating larger numbers of pollen grains in many diploid plant species, unreduced pollen grains can be detected on a small scale (at a level of ‰). Probably in the ES as well, some embryos may result from the development of cells at the normal ploidy level, and some from cells at a different ploidy level, see e.g. HAGERUP (1947) for diverse orchids, or the summary in LAKSHMANAN & AMBEGAOKAR (1984: 461-463). Thus, triploids may occur in



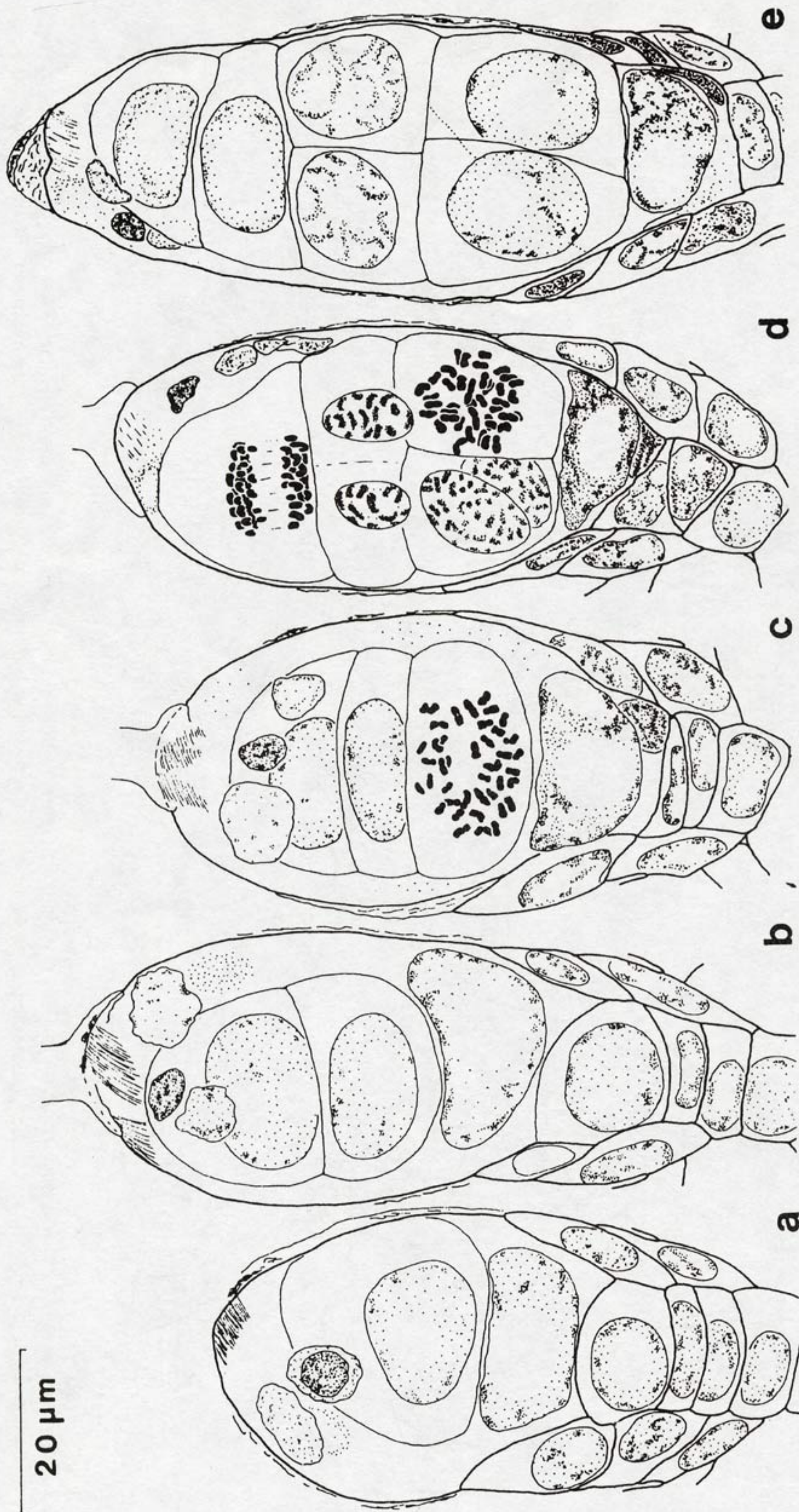


Fig. 1. *Nigritella rhellicani*, diploid with  $2n=40$  chromosomes. Nucelli with the first developmental stages of proembryos; a – zygote; b – two celled proembryo, basal cell (cb) above, apical cell (ca) below; c – three-celled stage, cb divided in ci and m, in ca metaphase plate of the first longitudinal division; d – proembryo with six cells; e – eight-celled proembryo, four cells on the tier of ca, two on that of m (in mitotic prophase). On the top of the embryo sac remnants of the filiform apparatus, the sperm nucleus (dark), vegetative nucleus and synergids, at the bottom the chalazal nucleus. Steiermark, Zirbitzkogel, Siegmundhütte.



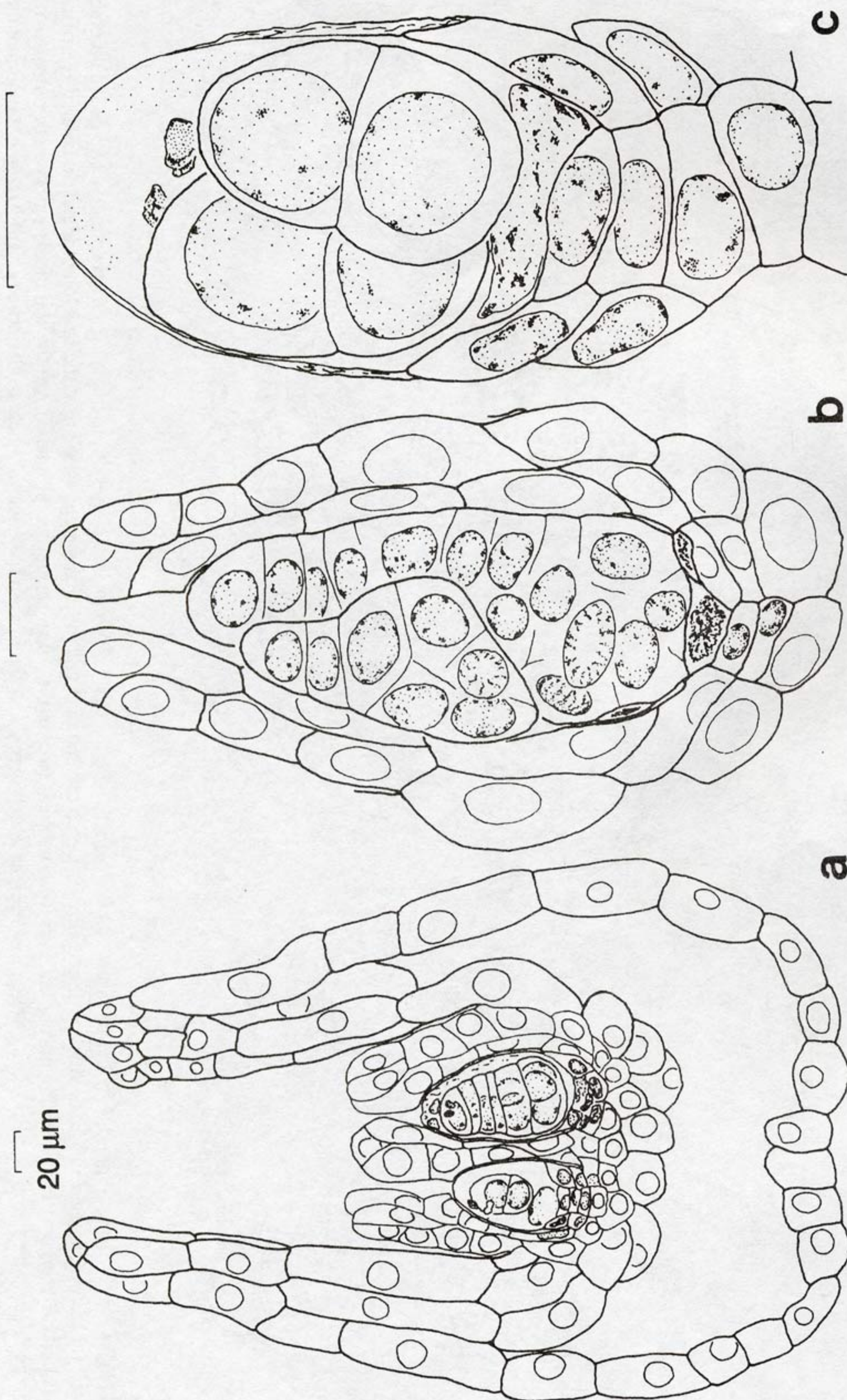


Fig. 2. Twins. a – twin ovules: two nucelli each with their own inner integument in a common outer integument; in the left a two-celled nucellus, in the right a ten-celled proembryo; b, c – twin embryos; b, c – two unequal proembryos in the nucellus and inner integument; outer integument not shown; c – two two-celled proembryos in the embryo sac. a, b – *Nigritella gabasiana* ( $2n=40$ ), Pyrenees, Formigal, 1530 m; c – *N. rhellicani* ( $2n=40$ ), Steiermark, Zirbitzkogel, Siegmundhütte. All scale bars represent 20 μm. Nuclei outside of the nucellus shown by their outlines only.



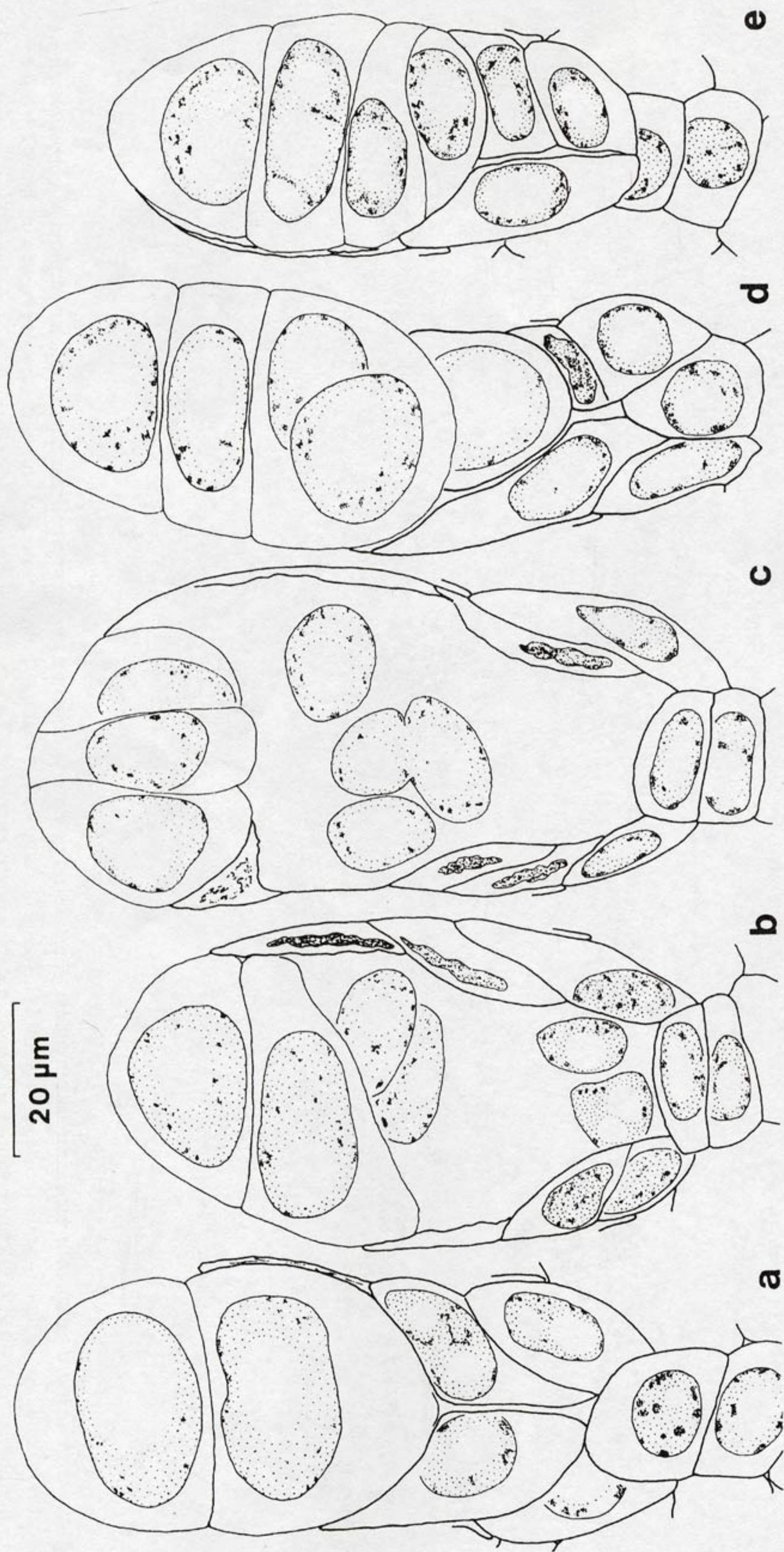


Fig. 3. *Nigritella nigra* subsp. *iberica* ( $2n=80$ ), first stages of development of nucellar embryos. Nucelli with two- to four-celled proembryos after very early degeneration of the EMC (a,d,e) and after initiation of ES development (adventitious embryos above degenerating four-nucleate embryo sacs: b,c); a,b – two-celled proembryos; c – three-celled proembryo in transversal position; d – four-celled proembryo (order of cells similar as in sexual ones) and a further embryogenic cell below; e – four cells of a proembryo in a linear row. Pyrenees: a,b Llanos del Ampriu; c,e Col du Pourtalet, 1790 m. From TEPPNER & KLEIN (1993: 197).



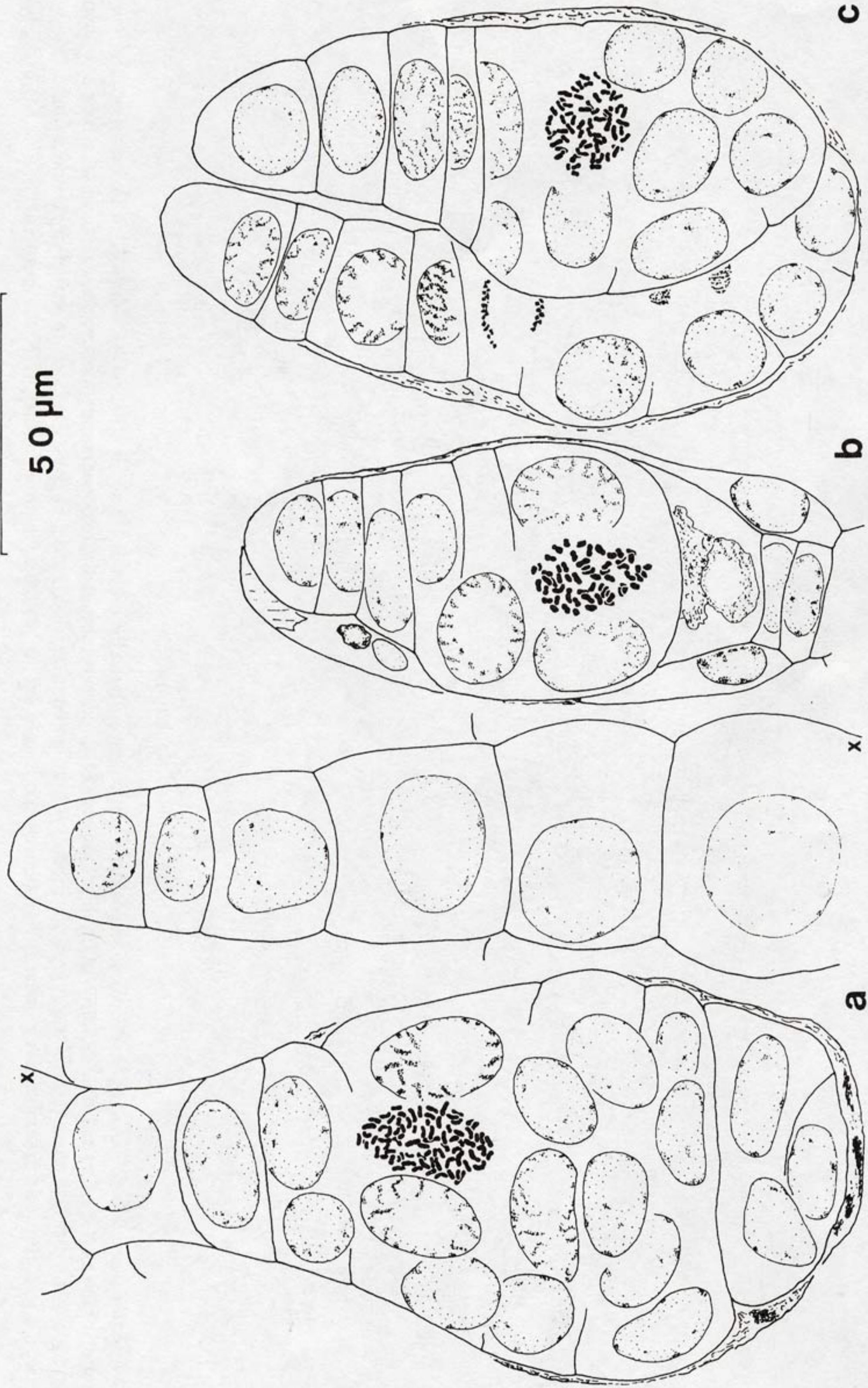


Fig. 4. *Nigritella nigra* subsp. *austriaca* ( $2n=80$ ), apomictic. Nucelli with proembryos out of a gynoeceum pollinated with pollen of diploid *N. corneliana*; a – two adventitious embryos (a small one at the base, most probably degenerating before maturity), the large one with a mitotic metaphase with  $2n=80$ ; b, c – triploid hybrid embryos with  $2n=57-59$  chromosomes in b and  $2n=62$  chromosomes in c. The second embryo in c is probably a twin because in competition with a sexual one an adventitious embryo should not have a basal position. Short lines in a symbolize the tips of the inner and outer integuments respectively. The cell walls which are parallel or sloping towards the optical plane are not shown in the figures of proembryos.



natural populations. Until now in *Nigritella* we have observed only one such spontaneous triploid in a population of diploid *N. carpatica* (TEPPNER et al. 1994: 180-181). At the time of megasporogenesis no embryogenic initials were present in this plant whereas they are already very prominent at this stage in apomictic taxa. Thus, this triploid was most probably not apomictic, but unfortunately the material available was from a field fixation and, therefore, no later stages could be observed. Such triploids may be important in the evolution of taxa with new chromosome numbers.

## Polyploids

Polyploids are mostly tetraploid with  $2n=4x=80$  chromosomes. Triploids are reported in *N. nigra* from Scandinavia (= *N. nigra* subsp. *nigra*; TEPPNER & KLEIN (1990)) and exist also in the Alps (but remain to be taxonomically investigated). A pentaploid taxon seems also to grow locally in the Alps (TEPPNER, unpubl.).

## Embryology

In spite of some meiotic anomalies the consequences are not spectacular. Pollen grains are normal but sometimes show small micronuclei (TEPPNER & KLEIN 1993: 195, 199). Through chromosome counts in the ES, full haploid numbers ( $n=40$ ) as well as small deviations from this chromosome numbers are detectable (TEPPNER & KLEIN 1985b: 321, 322, 1990: 20, 1993: 196, 200). In megasporogenesis and megagametogenesis, two extremes are possible. In many nucelli the embryo<sup>sac</sup> mother cells (EMC) degenerate very early before meiosis, giving rise to a very narrow nucellar space (Fig. 3a,d,e). In a few ovules, ES develop fully (observed in *N. widderi* (ROSSI et al. 1987: 136, 137) and in *N. nigra* (TEPPNER & KLEIN 1990: 20, 1993: 196, 200-201)). In most of the ovules EMC or ES degenerate at different stages of their development and then a large space in the nucellus remains (Fig. 3b,c). A variable number of ovules degenerates completely.

During pachytene or metaphase I, on the top or on the sides of the nucellus enlarged cells are already prominent in flower buds (without any necessity of a stimulus by a pollination event): these are embryogenic cells (embryocytes). They do not have the polarization of the zygote (compare e.g. NAUMOVA 1993: 24-27) and only in the case of early degeneration of EMC they have a relatively good central position in the ovule (Fig. 3a,d,e). The others lie at the periphery (Fig. 3b,c) and it is only during later development, that they are pushed in the nucellar space where the degeneration of ES proceeds. Development of nucellar embryos may be very irregular with respect to the sequence of divisions, the orientation, the dimension, the position and number of suspensors, etc. (TEPPNER & KLEIN 1993: 197-203). Even if more embryogenic cells are present in an ovule, usually only one or two embryos survive in the mature seed. Furthermore it is characteristic that, in a high percentage of seeds, the seed coat ruptures due to the rapid growth of the embryo(s).

As to the reasons of the irregular development of nucellar embryos I can only speculate. I believe that the main causes are the lack of polarization of the initials, poor insufficient orientation in relation to the physiological gradient in the ovule and, in many cases, the impediments caused by more than one embryogenic cell per nucellus. This view is supported by gynoecea in which the integuments degenerate early and the embryo initials must develop more or less freely in the ovarian space; in these cases orientation is greatly reduced and



irregularities increase dramatically resulting in very bizarre embryos with up to six suspensors (TEPPNER 1991b, TEPPNER & KLEIN 1985: 161, 163, 1993: 202-204).

### Partial sexuality?

As mentioned above for *N. nigra*, a small number of apparently fully developed ES was observed regularly. Are these functional and is this apomict consequently partially sexual? The easy way to test this is by pollination with pollen from an individual with a dominant marker character. However, analysis of progeny is not possible in *Nigritella* because these orchids cannot be grown from seed in the garden. For preliminary information I pollinated a tetraploid *N. nigra* subsp. *austriaca* with pollinia from diploid *N. corneliana*. Investigation of the first two gynoecia revealed nine proembryos containing one metaphase plate each suitable for chromosome counts. Seven of them were nucellar embryos with  $2n=80$  chromosomes, two of them were triploid hybrid embryos with  $2n=57-59$  and  $2n=62$  respectively (Fig. 4). The uneven numbers may be caused by the fact that not all parts of the plates were ideal for counting, or by meiotic anomalies, or both. Nevertheless, partial sexuality is apparently possible in *N. nigra*. But nothing can be said from this sample about the abundance (percentage) of sexual embryos, their viability, germination possibilities and importance in the populations. We have not found even one individual with deviating chromosome numbers in the field. At the moment we are trying to obtain more exact data and chromosome numbers for sexual hybrid embryos by growing them in vitro.

By production of adventitious (nucellar) embryos, female gametophytic stages are completely avoided in this mode of reproduction. Nevertheless, male gametophytes develop normally. The development of sexual female gametophytes is initiated in a high percentage of nucelli but only few of them, at least in *N. nigra*, achieve maturity and are functional.

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