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A PRACTICAL GUIDE TO BRYOPHYTE CHROMOSOMES

M.E. NEWTON

Pellia endiviifolia

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Chromosomes occur in every living cell of bryophytes, but are visible at the light microscope level only at certain stages of development and reproduction. They present an exciting means of combining field and laboratory studies which are concerned, not only with plant identification but also with population behaviour and evolution. Moreover, their study requires a relatively modest outlay beyond that required for a suitable microscope with a range of objectives that includes a $\times 100$ oil immersion lens.

Although this account is written in the knowledge that some bryologists wishing to look at moss and liverwort chromosomes do not have access to specially equipped laboratories, it is recognized that others do. Four staining techniques appropriate for use with a light microscope having no more than the basic accessories will therefore be described. One of them, that of acetic orcein or lacto-propionic orcein staining of unfixed spore mother cells for meiotic chromosomes, requires only one reagent, a stable and readily available stain, and is a realistic proposition for use at home. The other three involve preliminary fixation of material in a freshly prepared mixture of small amounts of absolute alcohol and glacial acetic acid. The former is highly inflammable as well as generally unobtainable, although there are substitutes which might be used. Glacial acetic acid, on the other hand, is obviously caustic. Both chemicals must be handled with care and do present something of a barrier to the practice of certain cytological techniques concerned with chromosomes but, for anyone who can overcome the problem, the rewards are great. Not only does it allow chromosomes to be seen at all times of the year and in species that never or rarely produce sporophytes, but it also opens the way to much more detailed study of the molecular and structural organization of bryophyte chromosomes. Nevertheless, even with access to the necessary chemicals, two of the three techniques to be described for mitotic chromosomes should not be contemplated for use outside a properly equipped laboratory. In this category are Feulgen staining and Giemsa C-banding, in view of the nature of some of the chemicals involved. They are, however, quite straightforward and well within reach of students and others with adequate facilities. For that reason, and also because an understanding of their purpose is among the best ways of ensuring a sound understanding of chromosomes in general and hence of worthwhile practical studies at whatever level they are practised, details of both are included. Indeed, it cannot be stressed too strongly that a sound understanding of the role of chromosomes as vehicles of genetic information is an essential prerequisite to studies of this kind, particularly as chromosome complements are more changing than number alone would suggest.

Each chromosome represents a single molecule of deoxyribose nucleic acid (DNA) together with a number of associated proteins, the histones and non-histones,

components which are referred to collectively as chromatin. The genetic information, which ensures continuity of form and physiology from one generation to the next but which is not completely immutable, is encoded within that DNA. There, in the double helical molecules, it undergoes a duplication prior to each of the cell divisions involved in growth to produce two exact replicas for transmission via the chromosomes to two daughter cells. They are events that are closely correlated with characteristically precise changes in the chromosomes.

THE CELL CYCLE

For much of the time, chromosomes remain invisible. They lie within a discrete part of the cell, the nucleus, which is separated from the surrounding cytoplasm by a porous nuclear envelope. The chromatin is in a relatively extended form, too fine for detection with the ordinary light microscope, and it is during this stage, known as INTERPHASE, that the genetic code is read and used in the manufacture of specific gene products. It is a process involving a large, more or less globular organelle, the nucleolus. Little else can be seen, but it is during the S-phase, or synthetic phase, of interphase that an exact copy of each molecule of DNA is made. Many cells proceed no further in nuclear terms than interphase, but differentiate into cells of various sorts depending on their location. There are clear distinctions, for instance, between the form and function of laminar cells, guide cells and stereids of moss leaves, or of photosynthetic cells and rhizoids of liverworts. Where active growth occurs, however, cell division will ensue and correlated with this are changes which render the chromosomes suitable for staining, thus making them visible throughout the process of nuclear division known as MITOSIS (Fig. 1).

Mitosis is a continuous process, but for convenience is divided rather arbitrarily into several stages. The first of these, PROPHASE, is marked by the breakdown of the nuclear envelope, the gradual reduction and disappearance of the nucleolus, and a progressive shortening and thickening of the chromosomes, each consisting of two parallel threads, the chromatids. A chromatid harbours one of the two copies of the DNA molecule. As prophase continues into PROMETAPHASE, the chromosomes are drawn towards the centre of a network, the spindle, of co-orientated microtubules which radiate, rather like lines of longitude, from each of the two poles of the structure to its widest point at the equator. The chromosomes reach alignment across the equator of the spindle at METAPHASE. They are held there by their attachment at a special site within each chromosome, the kinetochore, to two sets of shorter microtubules which extend only between one pole or the other and the equator. Those attached microtubules then serve to draw the two chromatids of each chromosome apart, taking them with trailing arms to opposite poles during ANAPHASE. At each of the poles, a daughter nucleus reforms as this, the TELOPHASE of mitosis, continues into interphase. The full complement of chromosomes, each now consisting of only a single chromatid, becomes progressively less well defined, the nuclear envelope reassembles and the daughter nuclei become isolated in two new cells by the production of an intervening cell wall.

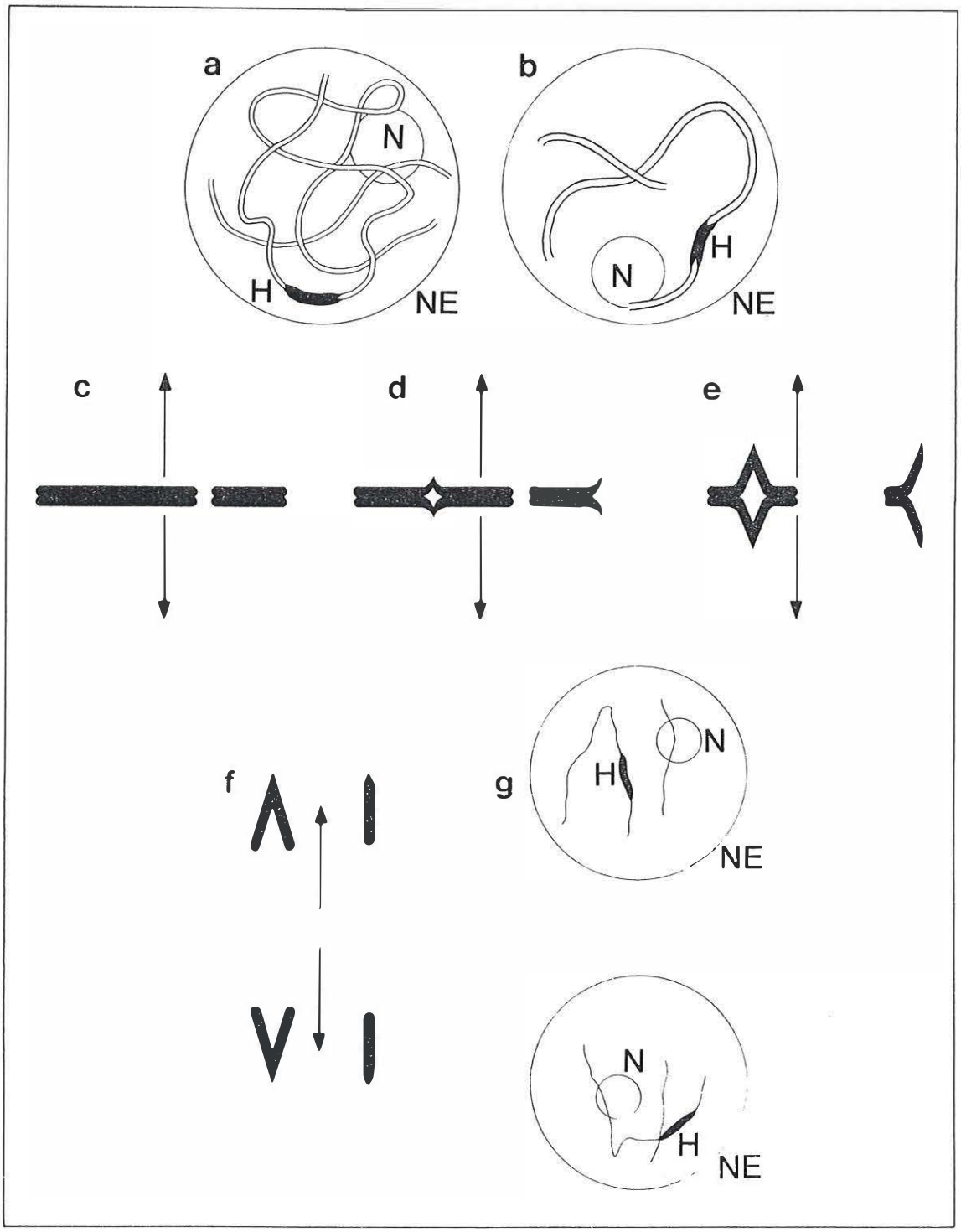

Fig. 1. Stages of mitosis. Two dissimilar chromosomes are depicted diagrammatically at (a) early prophase, (b) late prophase, (c) metaphase, (d) onset of anaphase, (e) mid-anaphase, (f) late anaphase and (g) telophase. (H=heterochromatin; N=nucleolus; NE=nuclear envelope). Arrows indicate orientation of the spindle axis and the direction of anaphase movement. For further detail, see text.

SLIDE PREPARATION FOR MITOTIC CHROMOSOMES

Preparation is straightforward, but requires one or two precautions to be taken. Since acetic acid and the stains used are caustic, anything, such as clothes and skin, with which they accidentally come into contact should be immediately rinsed in clean water. To protect the surface of the bench or table against spillages, it is advisable to use an off-cut of formica-covered board, which can be obtained quite cheaply. Other materials needed are as follows:

absolute alcohol

glacial acetic acid

acetic orcein or lacto-propionic orcein stain

tube of rubber solution (as used for bicycle tyre puncture repairs)

two glass specimen tubes with corks or polythene caps, but not rubber bungs

clean glass microscope slides (0.8 - 1.0mm thick)

clean square coverslips (22mm square, no. 1 $^1/_2$)

pad of blotting paper

pair of mounted needles (one slightly blunt), pair of forceps, fine scalpel.

Material for examination must be in an active phase of growth and is most conveniently taken from gametophytes. At least a fortnight before work is to begin, place a small amount of field collected material on its natural substrate in a jam-jar. Spray the contents liberally with tap-water and seal the jar with a sheet of polythene and a rubber-band. Place under illumination, but out of direct sunlight, protected from extremes of temperature; 16° - 18°C is ideal. Replenish the water by spraying whenever necessary to maintain a constantly humid atmosphere. When abundant growth is evident, chromosomes can be prepared for examination (Fig. 2).

1. Prepare fixative by placing about 12ml absolute alcohol in one specimen tube and 4ml glacial acetic acid in the other. Pour back and forth several times to facilitate mixing.
2. Immediately detach growing shoots, about 1cm long for ease of handling. Quickly blot away excess water on paper tissue, but do not allow material to dry. Immerse up to 20-25 shoots in the fixative. By dividing fixative between the two tubes, two different species can be dealt with simultaneously. Leave tubes overnight in cool place and, if necessary to store, subsequently in refrigerator.
3. Taking one shoot at a time, transfer first to 45% acetic acid in watch glass or small glass dish and then to drop of same on clean slide. It is helpful if the slide is placed on a black background under a dissecting microscope. Using a fine scalpel and mounted needle, dissect leaves away from the apex to reveal the tiny region of dense, white meristematic tissue, which is the site of repeated cell division. A similar block of tissue can be found in thalloid liverworts. The tissue, less than 1mm across, must not be allowed to dry as it awaits transfer to another slide.
4. The new slide must be scrupulously clean and free from fibres or dust. Using the point of a scalpel blade, lift the dissected apical meristem into a small drop of stain on the slide. Working on a white background, carefully lower a clean coverslip over the centre of the stain. Immediately grasp two corners of the coverslip to prevent lateral movement, locate the meristem and tap firmly with a blunt needle directly over the bryophyte tissue, and at that point of the coverslip only. This has

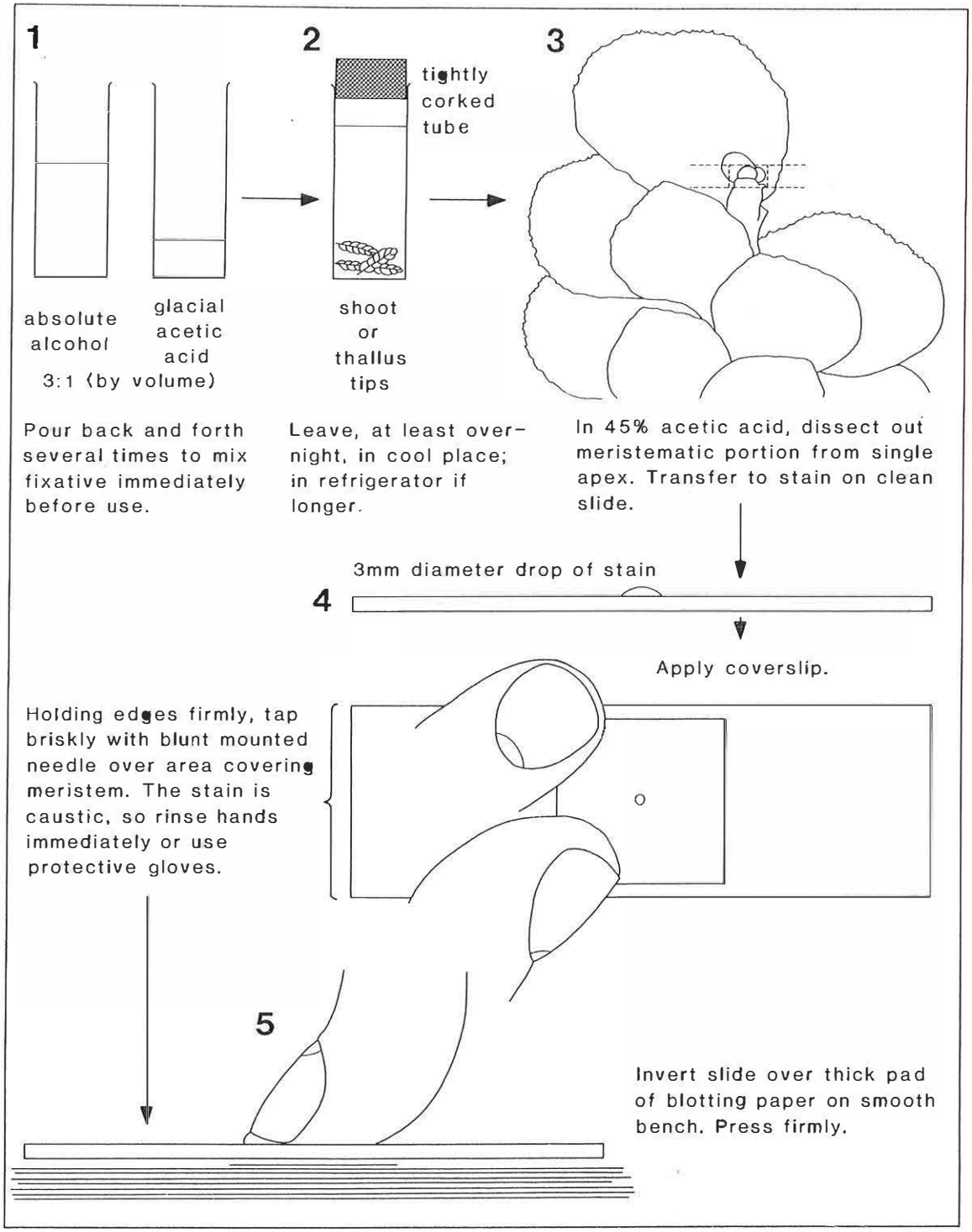

Fig. 2. Slide preparation for mitotic chromosomes. Numbers refer to schedule in text.

the effect of releasing the cell contents, together with the chromosomes, from inside the cell walls, but prevents excessive dispersal.

5. Invert the slide on a thick pad of blotting paper and press firmly, directly over the coverslip, with the thumb. Lateral movement must be prevented by holding the slide with the thumb and forefinger of the other hand.
6. Examine the slide and repeat the previous step as often as is necessary to secure a preparation in which all the chromosomes lie in a single plane. It is sometimes helpful to warm the slide very gently by passing it quickly a few times through a cool flame of a Bunsen burner or spirit lamp. Chance plays some part in this spreading, however, and it is usually pointless to persevere with any slide for more than two or three attempts to spread the chromosomes.
7. Seal the edges of the coverslip with rubber solution to protect satisfactory slides from evaporation. Stored in the freezing compartment of a refrigerator, such slides can be used for up to 7-10 days. They can be made permanent by any of the standard techniques for ultimate mounting in Euparal.

CHROMOSOME NUMBER AND MORPHOLOGY

An immense variety of chromosome numbers is found among mosses, ranging from $n = 4$ to $n = 66$, where n is used to denote the haploid or gametophytic complement. Some of those numbers, however, are much commoner than others. A significant proportion of species has $n = 5$ to 7, but many more, some 56%, lie within the range of $n = 10$ to 14 and are widely regarded as the result of duplication by polyploidy from ancestral forms with $n = 5$ to 7. Much higher numbers are similarly considered to be of polyploid origin but, superimposed on the basic pattern, there is evidence of numerical change involving less than a complete set of chromosomes. It is referred to as dysploidy or aneuploidy depending on the way in which it is believed to have occurred.

By contrast, chromosome numbers among liverworts are much more conservative. Almost 87% are of $n = 8$, 9 or 10, and polyploidy based on those numbers is comparatively rare. Moreover, there is little or no evidence of dysploidy and aneuploidy among liverworts.

It is general practice, in other organisms, to count and determine the structure of chromosomes at metaphase of mitosis when the position of kinetochores is marked as a constriction, the centromere, in each chromosome. In bryophytes, however, this is not usually feasible because the point of microtubule attachment in each chromosome becomes apparent, in many species, only when the chromatids begin to separate at that point at the onset of anaphase. Not until then is it possible to determine overall size of chromosomes as well as centromere position within each. Commonly used terms are indicated in Fig. 3, but greater precision can be applied by describing arm ratio, F%, whereby the shorter arm (p)is expressed as a percentage of the combined length of the two arms (p + q).

Both chromosome size and number obviously affect the amount of information that can be extracted from a chromosome preparation (Fig. 4). If all that is required is a knowledge of the number of chromosomes, it is worth noting that very small chromosomes are often extremely difficult to force into a single plane. Where they are numerous, too, counting is therefore particularly prone to error. In view of the dif-

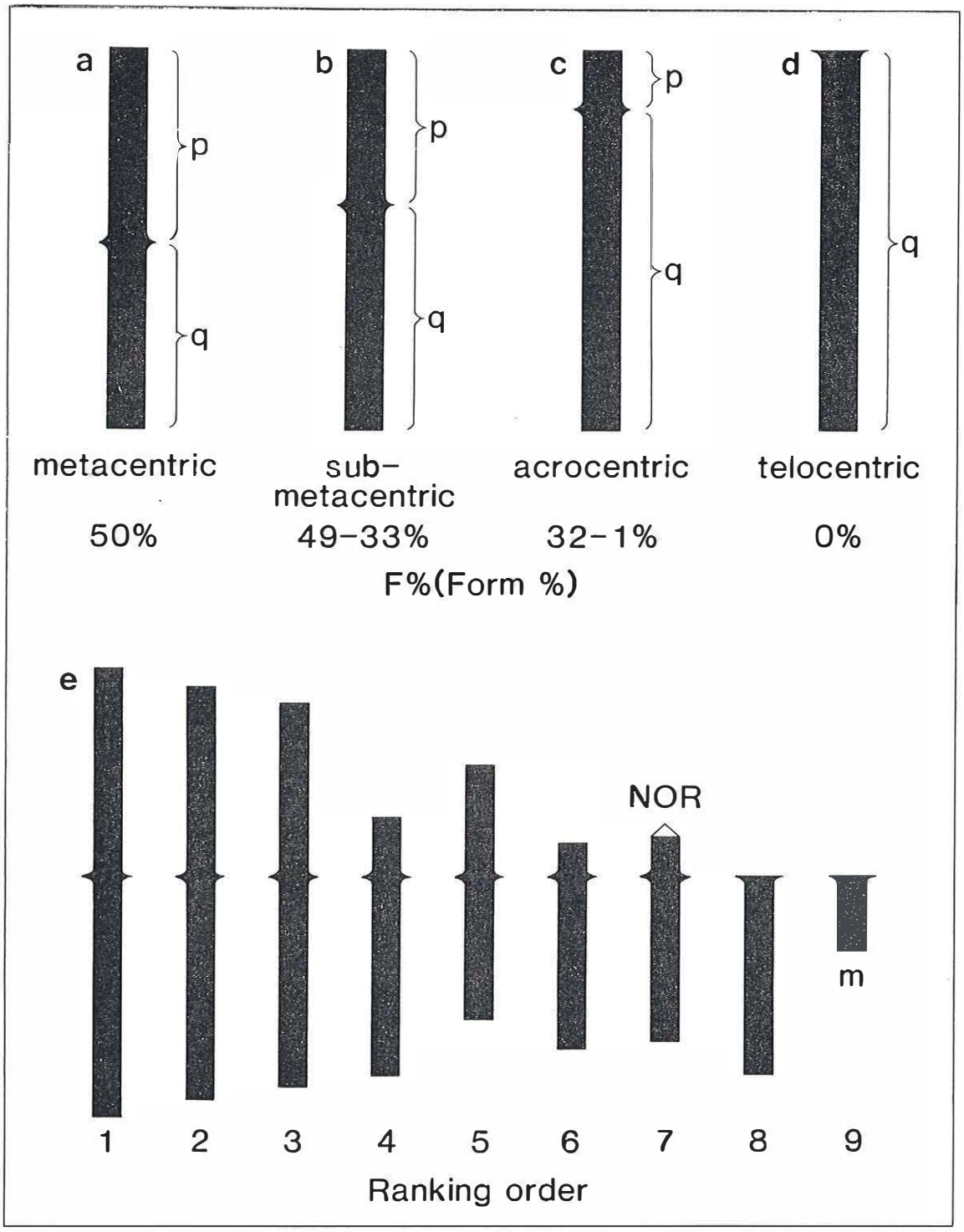

Fig. 3. Commonly used terms in bryophyte cytology. The range of F% is shown for (a) metacentric, (b) sub-metacentric, (c) acrocentric and (d) telocentric chromosomes, although these terms are often applied less precisely. Definition of (e) a complete set of chromosomes, the karyotype, is based on centromere position as well as ranking order and the location of a nucleolar organizer region (NOR) or regions, which are closely associated with the nucleolus during prophase. An m-chromosome is less than half the length of the chromosome immediately higher in ranking order.

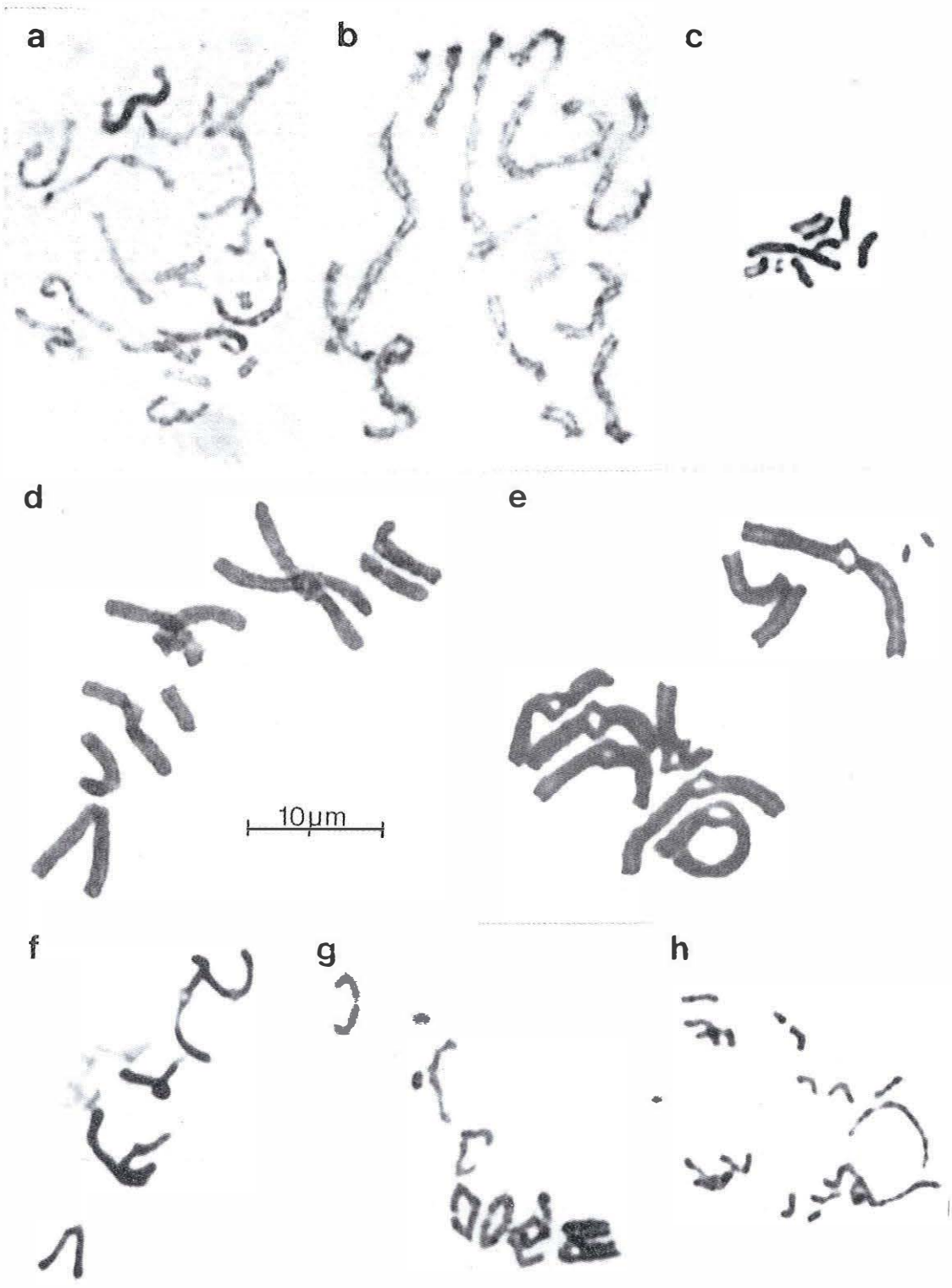

Fig. 4. Mitotic chromosomes after orcein staining in (a-e,g,h) liverworts and (f) a moss. (a) *Plagiochila britannica*, $n = 16 + 2m$, prophase showing heterochromatin; (b) *Pellia endiviifolia*, $n = 8 + 1m$, prophase almost devoid of heterochromatin; (c) *Plagiochila porelloides*, $n = 8 + 1m$, metaphase; (d) *Aneura pinguis*, $n = 10$, metaphase; (e) *Mylia anomala*, $n = 8 + 1m$, onset of anaphase; (f) *Plagiomnium ellipticum*, $n = 6$, onset of anaphase; (g) *Plagiochlla porelloides*, $n = 8 + 1m$, mid-anaphase; (h) *P. porelloides*, $n = 8 + 1m$, late anaphase.

ficulties imposed by small and/or numerous chromosomes, any of the following species would be ideal for initial studies:

Pellia epiphylla	*Mnium hornum*
P.neesiana	*Plagiomnium* spp
P.endiviifolia	*Rhizomnium punctatum*
Mylia spp.	*Pleurozium schreberi*

THE LIFE CYCLE

Although, for ease of handling, mitotic chromosomes of bryophytes are usually studied in meristematic tissue of gametophytes, there are many other possible sources of suitable material (Fig. 5). They include developing protonemal buds and other sites of vegetative propagation, as well as early stages of antheridial and archegonial development. Well-synchronized mitosis prior to spermatogenesis makes young antheridia a particularly rich source of material for this purpose. Once fertilization has occurred, however, other factors must be borne in mind because the sporophyte is diploid with two sets of chromosomes, one from its male parent and the other from the female. If self-fertilization occurred in a monoecious species, these would, of course, be one and the same. In mosses, mitotic chromosomes are readily found at tips of immature setae prior to capsule expansion. In liverworts, however, they are seen before extension of the seta but while the developing capsule is still green. Slide preparation at each of these stages is exactly like that described for shoot and thallus apices, with the sole proviso that the temptation to use large pieces of tissue should be resisted, because it prevents adequate spreading of chromosomes. It is better to divide the total available into several pieces for use on a number of slides.

In addition to extending the availability of mitotic chromosomes, the sporophyte generation of bryophytes presents a transient but nonetheless extremely profitable phase for chromosome study, particularly in mosses. It is the stage at which the diploid number of chromosomes is halved for a return, via spores, to the gemetophytic part of the life cycle. The process by which it is achieved, MEIOSIS (Fig. 6), is also the means by which the original parental chromosomes and genes are rearranged in different combinations, providing the sporophyte arose from outcrossing rather than self-fertilization.

As chromosomes emerge from the interphase immediately preceding meiosis, they enter a prolonged prophase, the first stage of which is referred to as LEPTOTENE, when the chromosomes, diploid in number, are still extended. ZYGOTENE begins as homologous chromosomes, one from each parent, become associated, initially at a few points but ultimately coming to lie closely parallel to each other throughout their entire length. Indeed, they are physically linked by an intermediary synaptonemal complex, thus establishing the haploid number of paired chromosomes, each coupled pair being referred to as a bivalent. Some shortening and thickening has occurred by PACHYTENE, but the main events of this stage are not seen at the light microscope level. They concern the formation of chiasmata at one or more points in each bivalent whereby, at each of these points, one chromatid of each component homologue is involved in a reciprocal exchange. It is by this means that genetic recombination, a rearrangement of genetic information within chromo-

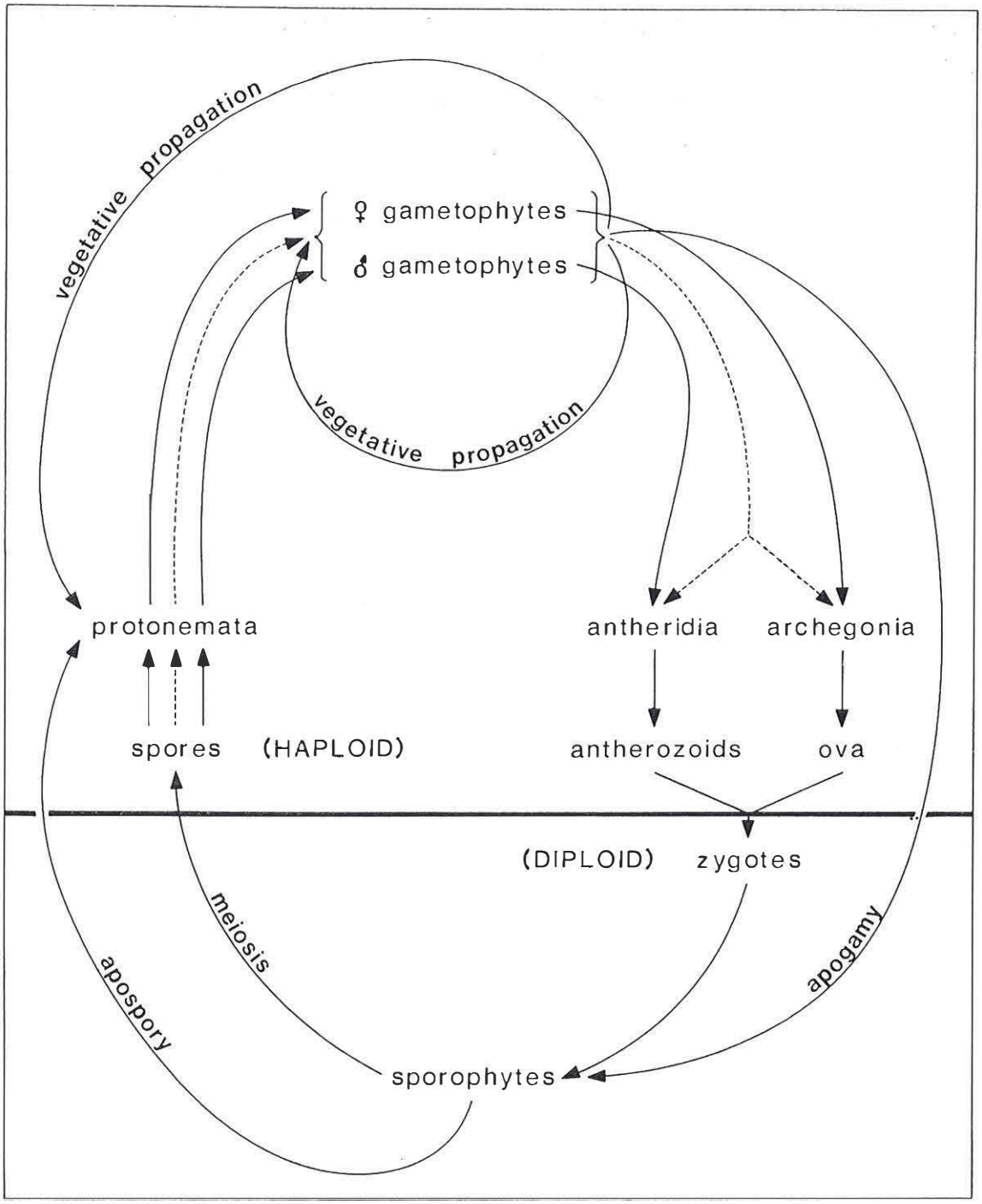

Fig. 5. The bryophyte life cycle. The gametophytic chromosome number (haploidy) is doubled (to diploidy) by zygote formation and is restored by meiosis. These processes may be bypassed by apogamy or apospory, respectively, which involve a change of generation, but with constancy of chromosome number.

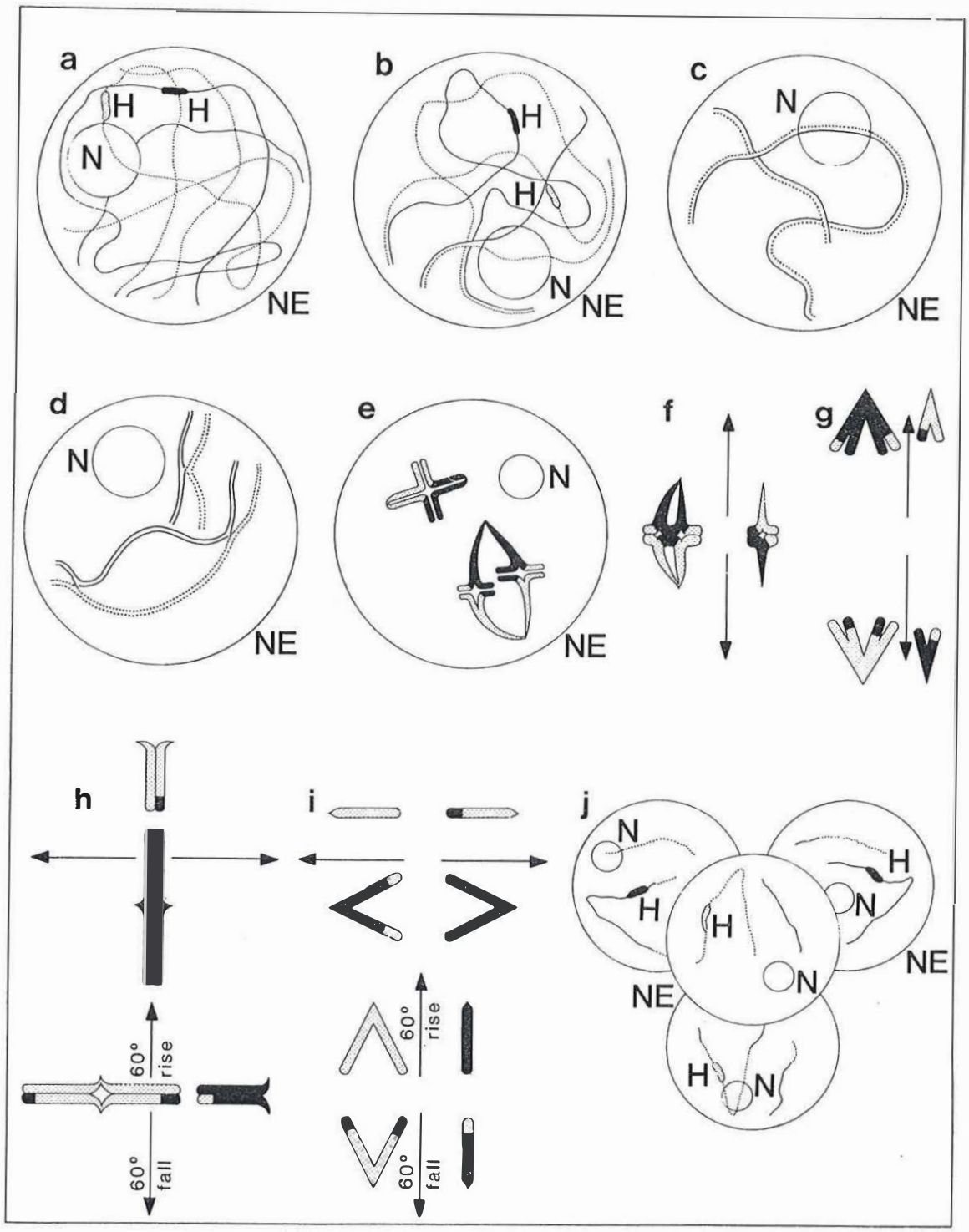

Fig. 6. Stages of meiosis based on the same two diagrammatic chromosomes as Fig. 1, but now in a diploid nucleus, one set having been acquired via a male gamete (antherozoid) and the other homologous set via an ovum. Four chromosomes are therefore involved. The two parental sets are distinguished by solid and stippled representation during (a) leptotene, (b) zygotene, (c) pachytene, (d) diplotene, (e) diakinesis, (f) metaphase-I, (g) anaphase-I, (h) onset of anaphase-II, (i) late anaphase-II and (j) telophase-II. Symbols as in Fig. 1.

somes, is brought about. As the bivalents enter DIPLOTENE, the synaptonemal complexes disperse, resulting in a lapse of the close pairing between homologues. The only remaining points of contact are those where chiasmata occur. A further period of condensation follows as the bivalents assume the highly condensed form characteristic of DIAKINESIS, the stage preceding their alignment across the equator of a spindle at METAPHASE-I. During ANAPHASE-I, which follows, the component homologous chromosomes are drawn apart to opposite poles. This is in direct contrast to the situation at anaphase of mitosis, which involves the separation of the chromatids making up each chromosome. Depending on their orientation at metaphase-I, in relation both to the poles and to other bivalents, it is therefore evident that anaphase-I represents a further opportunity for rearrangement of genetic information. However, unlike that which depends on chiasma formation during pachytene and involves changes within chromosomes, this at anaphase-I is between whole chromosomes and is referred to as independent assortment. There follows a short and generally incomplete TELOPHASE-I, which passes directly into PROPHASE-II. This prophase closely resembles the equivalent stage of mitosis, in common with the succeeding METAPHASE-II, ANAPHASE-II and TELOPHASE-II and their counterparts in mitosis, after which, chromatid separation having occurred, four nuclei become incorporated in a tetrad of developing spores.

SLIDE PREPARATION FOR MEIOTIC CHROMOSOMES

With a little practice, it is quite easy to recognize moss capsules in which meiosis is occurring. In nearly all genera and species, they have reached their mature size and shape and are a translucent green. In addition, there is often a very faint and/or incomplete ring of some shade of red or brown at the base of the operculum. Meiosis is more or less synchronous within a capsule in many species and, as soon as metaphase-I has passed, the capsule becomes suffused with an opaque, yellowish tinge. Although, with the exception of the brown ring, similar changes occur in liverwort capsules, it is rather more difficult, but not impossible, to select the right ones for meiosis because they are usually concealed by a perianth or involucre. Liverworts are also less easy to handle than mosses because, except for the Marchantiales and Sphaerocarpales, meiosis involves a nucleus lying at the centre of a four-lobed spore mother cell, which is extremely difficult to flatten. In mosses, however, the nucleus is situated in a more or less globular cell, although it is somewhat quadrate in the Polytrichales.

A satisfactory technique for slide preparation involves the use of fresh material, kept moist until required, but suitable capsules can also be fixed in three parts of absolute alcohol to one of glacial acetic acid if necessary. Fixed capsules should be transferred to 45% acetic acid before proceeding as for fresh material, as follows. The only equipment additional to that for mitotic preparations is a short brass rod with a flat end, about 3.5mm square or 4mm diameter if round.

1. Place a small drop of stain at the centre of a clean slide. Towards the end of the same slide, dissect out the entire contents of a capsule and transfer quickly to the stain. Do so by holding the seta with a pair of forceps while making two cuts with a sharp scalpel; one to remove the operculum or, in cleistocarpous species, the

apex from the capsule and the other a longitudinal cut in the capsule wall. Clear all the debris from the slide.

2. Macerate the material in the stain by tapping repeatedly with a brass rod.
3. Working on a white background and using two mounted needles, lift any large pieces of debris out of the stain and discard.
4. Apply a coverslip to the drop of stain. Invert slide on a pad of blotting paper and apply firm pressure with the ball of the thumb in the area of the coverslip.
5. Examine the slide and apply further pressure if necessary to obtain perfectly flat preparations.
6. Seal the edges of the coverslip with rubber solution. As with mitotic preparations, the useful life of slides can be prolonged for 7-10 days by storing in the freezing compartment of a refrigerator, and slides can also be made permanent.

MEIOTIC CHROMOSOMES

The degree of chromatin condensation, although less than that in mature spermatozoa, is much greater during meiosis (Fig. 7) than mitosis. Even where very large numbers of chromosomes are involved, the bivalents of diakinesis and metaphase-I are therefore clearly discrete structures and counting them is a relatively straightforward procedure, since overlapping is a less serious drawback than it is with mitotic chromosomes. Valuable behavioural information can also be obtained, although discernible structural detail is necessarily limited in all but a few genera. Notable exceptions include members of the Mniaceae, particularly *Mnium* spp. and *Plagiomnium* spp., in which centromeres as well as the positions of chiasmata are clearly defined. Bivalent size is not, however, the only factor determining the usefulness of meiotic preparations in structural studies. There are many taxa in which large chromosomes offer no more insight into bivalent morphology than do others with small chromosomes. *Polytrichum* spp. and *Hookeria lucens* in the first category, for instance, are no more informative in this respect than are the much smaller bivalents of *Tortula* spp. Because of this, it is particularly important to establish that metaphase-I chromosomes of such taxa are indeed bivalents, the existence of which is dependent on the presence of at least one chiasma in each. Serious counting errors may otherwise result from relatively minor fluctuations in environmental factors that are reflected in a reduced chiasma frequency and, hence, in the presence of unpaired chromosomes, univalents, at metaphase-I.

CYCLICAL CHANGES IN THE AMOUNT OF DNA

Acetic orcein and lacto-propionic orcein, the stains recommended so far, are acidophilic or acid-loving. They therefore stain DNA and the non-histone, acidic proteins of chromatin, as well as any associated ribose nucleic acid (RNA) there may be. The surrounding cytoplasm also takes up stain to a certain, though lesser, extent.

An alternative method of staining that is sometimes used for bryophyte chromosomes but rarely exploited to its full potential involves the Feulgen technique using leuco-basic fuchsin, which binds specifically to critically hydrolysed DNA. The chro-

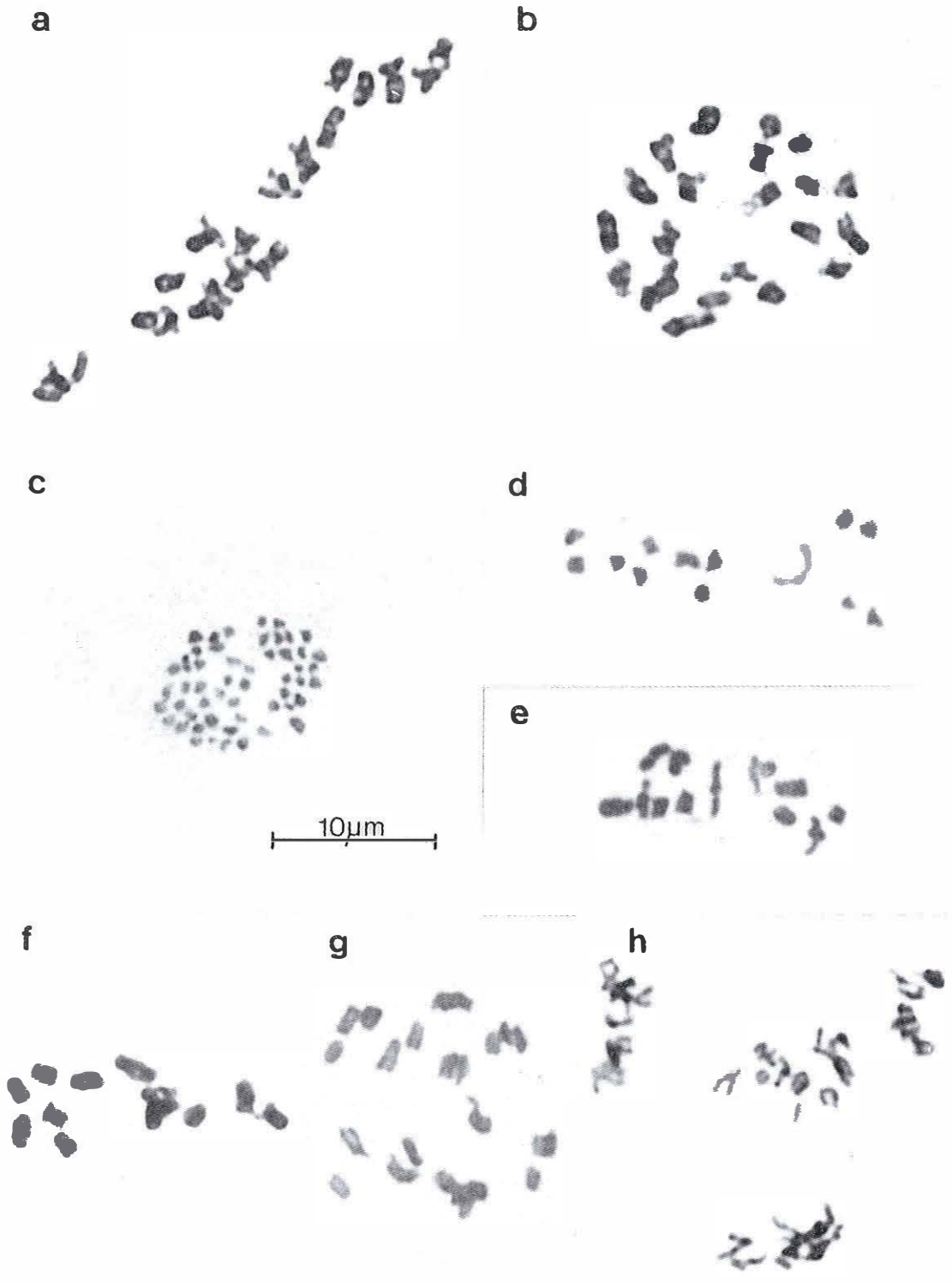

Fig. 7. Meiotic chromosomes in the mosses (a) *Atrichum undulatum*, *n* = 14, diakinesis; (b) *A.undulatum*, *n* = 21, metaphase-I; (c) *Tortula brevis*, *n* = 52, metaphase-I; (d) *Gymnostomum aeruginosum*, *n* = 13, metaphase-I; (e) *Dicranum tauricum*, *n* = 12, diakinesis with two univalents (above left); (f) *Hookeria lucens*, *n* = 12, metaphase-I; (g) *H.lucens*, mid anaphase-I; (h) *Atrichum undulatum*, late anaphase-II.

mosomes become deeply stained in proportion to their DNA content and lie in a completely colourless background. This is in itself an advantage over orcein but, when carefully standardized, also provides a means of monitoring the amount of DNA in different cells throughout the life cycle. There are certain cells, such as those of moss protonemata, for instance, in which successive rounds of replication are not separated by the intervention of mitosis in the normal course of events. Although measurements of this sort require access to special equipment and are beyond the scope of this account, Feulgen staining is still a worthwhile alternative technique for producing chromosome preparations of remarkable clarity. It is, moreover, a convenient method, requiring only the following additional materials:

5N hydrochloric acid (HCl)

leuco-basic fuchsin

additional glass specimen tubes with corks

disposable protective gloves.

1. Fix gametophytic apices in freshly mixed 3:1, absolute alcohol: glacial acetic acid as previously described.
2. Transfer material to tube of tap water for rinsing.
3. Remove excess water from the gametophytes by blotting before placing in a tube of 5N HCl for hydrolysis at room temperature (c. 20°C) for 30 minutes. Alternatively, 1N HCl at 60°C for 6 - 8 minutes will produce the same results but, in either case, the treatment must be precise. It will render the material extremely soft, so that subsequent handling must be particularly careful.
4. Pour the hydrochloric acid away and rinse in tap water briefly before replacing with leuco-basic fuchsin for 30 minutes at room temperature.
5. Transfer gametophytes to tap water immediately prior to slide preparation, carried out in accordance with steps 4 to 7 of the schedule for mitotic chromosomes, but substituting a small drop of 45% acetic acid for the drop of orcein stain.

CHROMATIN VARIATION

Superimposed on the normal sequence of replication, condensation, division and decondensation of chromatin, is asynchronous behaviour that was described first in liverworts but which is now known to be widespread among plants and animals. It involves those segments of chromatin for which the term heterochromatin was coined to distinguish them from the majority of chromatin, the euchromatin. They are identified by the fact that they remain condensed throughout the greater part if not all of interphase. Their recognition is therefore a straightforward matter in orcein as well as Feulgen stained preparations. In fact, it provides a convenient means of discriminating between the morphologically similar but cytogenetically distinct liverworts *Pellia epiphylla* ($n = 9$) and *P. borealis* ($n = 18$), which have one and two large blocks of heterochromatin, respectively.

More sophisticated techniques of chromosome treatment for use at the light microscope level are capable of probing more deeply into the underlying molecular composition of heterochromatin. As a result, it is now known that heterochromatin is of two basic types depending on the nature of its constituent DNA. One, which is facultative, differs from euchromatin in that its condensation is out of phase in cer-

tain cells and tissues but not in all. Detailed biochemical analysis in other organisms than bryophytes has shown that its DNA resembles that of euchromatin. The second broad category of heterochromatin, however, is constitutive and includes extensive tracts of DNA that are not part of the genetic code, which is composed of specific ordering of the purine (adenine and guanine) and pyrimidine (thymine and cytosine) bases within the double helix. Instead, extensive repetition of apparently meaningless sequences is the hallmark of constitutive heterochromatin.

Giemsa C-banding has proved to be an effective means of locating those highly repetitive sequences within chromosomes and, hence, of discriminating between facultative and constitutive heterochromatin during interphase, as well as between constitutive heterochromatin and euchromatin at those stages of mitosis and meiosis when they are equally condensed. It is also possible to discover much more about the underlying nature of those molecular differences by using DNA-specific fluorochromes, of which there are many, differing from each other in several points of detail. The two that have so far been applied to bryophytes, quinacrine dihydrochloride and Hoechst-33258, are ones which bind specifically to concentrations of adenine-thymine base pairs in DNA. Illuminated at an appropriate wave-length, they then fluoresce with greater or lesser intensity according to the degree of clustering of AT base pairs, and hence of the amount of bound fluorochrome, along the length of the chromosomes. The method, albeit technically simple, requires special microscope facilities which place it outside the scope of this account. Giemsa C-banding, however, offers an exciting insight into the molecular conformation of chromosomes that handsomely repays the time and effort involved in a rather prolonged, though not difficult, staining procedure.

GIEMSA C-BANDING

Because slide preparation and staining extends over at least three days, those points at which it is feasible to interrupt the sequence are indicated below.

1. Gelatine coated slides must be prepared beforehand but can be stored indefinitely. To do so, place clean, washed slides vertically in a plastic or wire rack for dipping in the following solution, prepared with gentle heat and continuous stirring but allowed to cool to room temperature before use. The slides, still in the rack and protected from dust, take about 24 hours to dry.

   1g powdered gelatine

   0.1g chrome alum

   200ml distilled water.

2. Fix and prepare gametophytic material according to the method described for orcein preparations of mitotic chromosomes but making two modifications:
   (i)the slides used should be gelatine coated slides, care being taken to prevent damage to the delicate coating, which is intended to act as an adhesive, causing the cells and chromosomes to adhere to the glass surface.
   (ii)instead of a drop of stain, use a drop of 45% acetic acid.
   It is desirable to accumulate slides for subsequent treatment in batches of ten, which can be kept for up to 24 hours in the freezing compartment of a refrigerator, if necessary, before proceeding further.

3. After peeling away the dried rubber solution, prise off each coverslip with a razor blade. Do so while the slide is still deeply frozen from immersion in liquid nitrogen or from the application of solid carbon dioxide. Allow the slides to dry overnight at room temperature in a dust-free atmosphere.
4. Prepare modified Hanks' solution as follows. Heat is not required. Store in a refrigerator until required.

   8g sodium chloride (NaCl)

   1g glucose

   0.4g potassium chloride (KCl)

   0.35g sodium hydrogen carbonate ($NaHC0_3$)

   0.6g potassium dihydrogen orthophosphate (KH_2PO_4)

   0.048g di-sodium hydrogen orthophosphate (Na_2HP0_4)

   1ml phenol red (1% soln)

   distilled water to 1 l.
5. Within 24 hours of removing the coverslips from the slides, place ten of them in pairs back to back in a Coplin jar of 0.2N HCl for 1 hour at room temperature.
6. Meanwhile place two empty Coplin jars, with lids, in a water bath at 50°C, and one in a bath at 60°C, the last jar to be filled with Hanks' solution. It is advisable to warm the jars gently beforehand in order to prevent their cracking.
7. Prepare a 5% solution of barium hydroxide (with heat and constant stirring) and filter it into one of the Coplin jars in the 50°C bath.
8. After 1 hour, transfer slides directly from 0.2N HCl to the empty jar in the 50°C bath. With a paper tissue, wipe the scum from the surface of the barium hydroxide solution and pour the solution over the slides in the otherwise empty jar. Leave for 1.5 to 3 minutes depending on the species, the exact time to be found by trial. Immediately flush away all barium hydroxide by placing the jar under a running cold water tap.
9. Transfer the slides to Hanks' solution at 60°C for 1 hour.
10. Meanwhile, prepare a 2% solution of Giemsa stain in phosphate buffer at pH 6.8. (Buffer tablets can be obtained for the preparation of 1 litre of solution). Place in clean Coplin jar.
11. After 1 hour in Hanks' solution, rinse slides in jar under the cold water tap. Transfer the slides to the jar of buffered Giemsa stain for 20 - 30 minutes at room temperature.
12. Rinse the slides individually in pH 6.8 buffer solution. Shake off excess buffer and leave slides to dry on very gentle heat on a plate warmer. Mount in Euparal or DPX mountant using clean, dry coverslips.

C-BANDS AS CYTOLOGICAL MARKERS

The value of C-bands as cytological markers is related to their considerable specificity within and between chromosomes. Otherwise similar sets of chromosomes in different species may be distinguished by their distribution of C-bands and, where they are sufficiently numerous, the bands may also serve to identify individual chromosomes (Fig. 8). With the exception of *Pellia endiviifolia*, in which there are few C-bands, the genus *Pellia* is particularly relevant in this context. Being known to re-

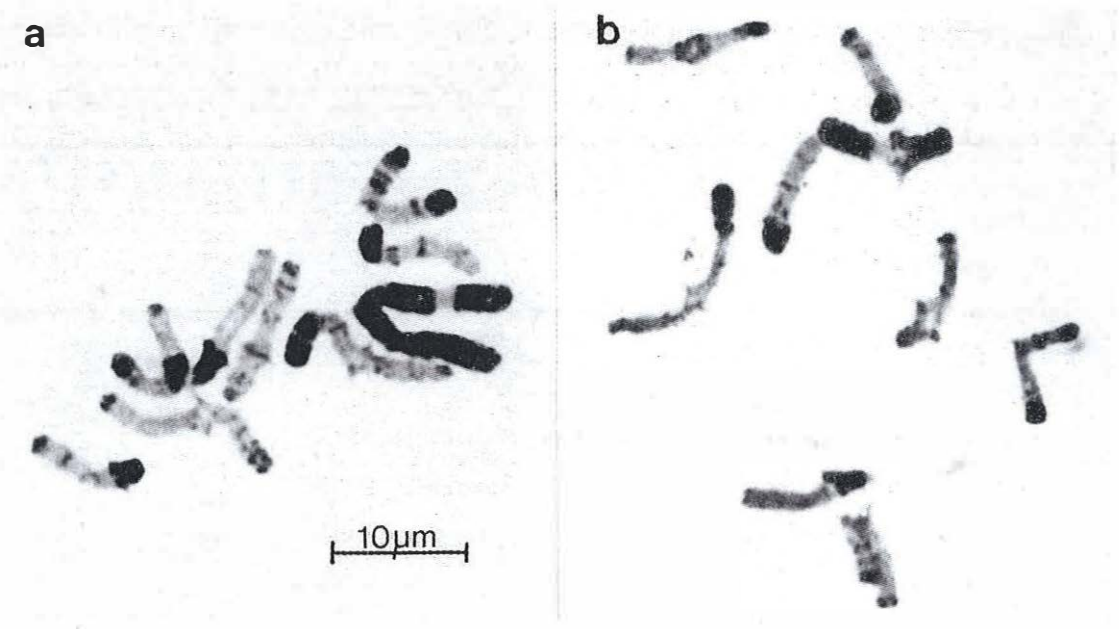

Fig. 8. C-banded gametophytic mitotic complements of *Pellia neesiana* (n = 8 + X/Y) at metaphase about the onset of anaphase, showing (a) the female-specific X-chromosome (right) and (b) the male-specific Y-chromosome (second from top centre).

spond well to the staining technique described, it can be relied on to produce consistently encouraging results.

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