

16. Standardized protocol for cytological study of meiotic cells in plants using basic acetocarmine staining technique

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Abstract:

Plants have very different shapes and genetic makeup. Some species have only a few chromosomes, while others have a lot more. This is another example of how evolution works. Differences in chromosome number provide important insights into how new plant species arise and adapt. Polyploidy, which means that a plant has more than one set of chromosomes, is one reason for this difference. This can lead to the creation of new species. This process has been especially important for the success of many flowering plants and ferns in terms of evolution and diversity. Counting chromosomes is still one of the easiest and most powerful ways to learn more about these genetic changes. In contrast to advanced DNA sequencing methods, this classic cytological technique requires basic microscopy and staining to visualise a plant's karyotype, providing a direct view of its genetic structure and evolutionary history.

Introduction

Plants come in all shapes and sizes and with wildly different genome setups.

Some have very few chromosomes, just with 4 per cell in certain tiny flowers, while others carry the record-breaking number of a fern (*Ophioglossum reticulatum*) with **1,440** chromosomes. These extreme differences matter; when a plant ends up with extra chromosome sets, it often becomes a new species. In fact, studies estimate that about **15%** of flowering plant speciation events and **30%** of fern speciation events involve whole-genome duplications. Moreover, genomic analyses show that whole-genome duplications have happened repeatedly throughout plant evolution, helping drive the incredible diversity of green plants today. In short, knowing a plant's chromosome number and how its chromosomes pair up during cell division gives botanists a powerful clue to its evolutionary history and traits (Leebens *et al.*, 2019).

Chromosome numbers can therefore help answer big questions: Why does species *A* look and behave differently from its close relative *B*? Did genome duplication spark a new lineage? Is an odd form of a plant really a separate species? Unlike DNA sequencing, which reads genomes in detail,

chromosome counting offers a direct peek at the karyotype, the physical arrangement of an organism's genome. For example, two plant lineages might have the **same** number of genes and hence similar "genome size" but differ in chromosome count; only a cytogenetic squash reveals that difference. Such cases are known, e.g. the horsetail

plants (*Equisetum*) have the exact chromosome count ($2n = 216$) despite one clade having double the DNA content of its sister group. Without actually counting chromosomes under the microscope, researchers would wrongly assume different ploidy levels. (Windham *et al.*, 2020)

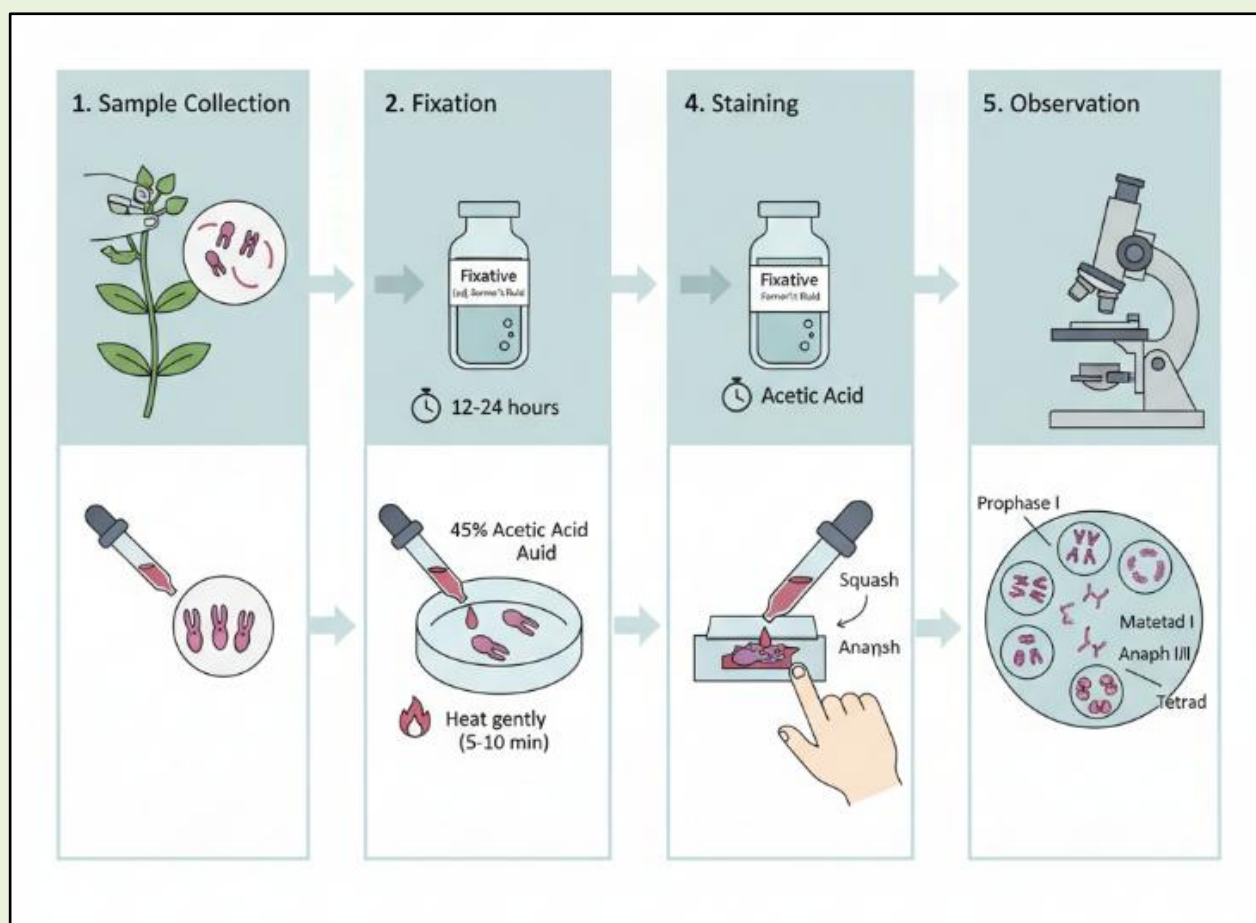


Figure 1: Steps in cytological observation

Why Count Chromosomes?

a) Track species origins:

Counting chromosomes can pinpoint recent speciation via polyploidy. A sudden doubling of chromosome number often **creates instant reproductive isolation**, allowing new species to form.

b) Calibrate genome measurements:

When comparing DNA amounts (by flow cytometry or sequencing) across species, chromosome counts serve as a ground truth. Without them, one might misinterpret large-genome species as polyploid.

c) Reveal hidden variation:

Some plants show *intraspecific* chromosome-number differences (different populations of the *same* species have different counts). *Carex* sedges, for instance, can have multiple cytotypes with very different karyotypes, but almost the same DNA amount; only by direct counts can this be seen.

d) Study evolution:

Ancient whole-genome duplications leave a mark on chromosome arrangements. Cytogenetic data (paired chromosomes and their shapes) complement DNA data for reconstructing plant evolutionary trees.

e) Low cost:

For many botanists, classic squashes are nearly free to do. The only specialised equipment needed is a microscope.

f) Widespread applicability:

From grasses to orchids to ferns, the squash method works in most plant groups. When done correctly, virtually any flowering plant can be sampled for chromosome number.

Simple Cytogenetics Protocol

A classic chromosome squash is not as complicated as it sounds. The goal is to flatten cells at just the right stage for

meiosis so the condensed chromosomes can be counted. Here is an overview of the process (Manoj, 2022).

1) Collecting young buds:

Young flower buds of appropriate size must be collected from the plants between 6.00 AM and 11.00 AM on a bright, sunny day, when pollen cells are dividing. For many plants, that means gathering small flower buds. Start with the tiniest buds available, in a range of sizes, because only some cells will be in mid-division. Every 15 minutes, the cell stage may change during prophase I of meiosis. We have to observe accordingly: if pachytene is found but we need zygotene, we need to collect buds 15 minutes earlier. First, we have to check for standard hours, then collect buds every 15 minutes. Next, we have to standardise the timing of genotypes for each genotype. Bud collection timing and temperature are recorded for the location using a digital thermometer. Bud size can be calculated using vernier callipers. We need to standardise bud collection timing to match the bud size. For every season, cell stage times may change, so we need to

standardise the protocol accordingly.

2) **Fixation of the tissue:**

Fixation is an important process for the sudden termination of cell division, enabling the study of different stages of cell division in detail. Properly fixed buds can be stored in a freezer for months or years. Carnoy's II solution has to be prepared in the morning, as its alcohol concentration will evaporate if prepared in the evening. Young floral buds have to be fixed in Cornoy's II fixative, which is composed of six parts of absolute ethyl alcohol, three parts of chloroform and one part glacial acetic acid (6:3:1) for 24 hrs and then washed thoroughly with 70 per cent ethyl alcohol until all residues of Cornoy's II fixative are removed. After 24 hours, these are transferred to 70 per cent ethyl alcohol and stored at 4°C for further studies.

3) **Preparation of Aceto-Carmine stain:**

Aceto-carmine stain is commonly used for chromosomal differentiation in meiotic studies; usually, 1 per cent is desirable, and it is prepared by adding 1 gram of certified carmine powder to 100 ml

of boiling 45 per cent glacial acetic acid. After rapid cooling, the stain is filtered, and into that drop or two of ferric chloride is added to affect colouring, and the mixture is stored for further studies.

4) **Preparation of PMC smears using the squash method:**

Young floral buds stored in 70% ethyl alcohol are washed thoroughly with distilled water four to five times to remove all traces of alcohol. Under a dissection microscope, the five anthers are separated from the floret, and one anther is placed on a clean glass slide. Try to remove extra flower parts so you have mostly anthers in the fixative. A drop of 1% acetocarmine stain is added, and the anther is gently tapped and squashed with a sharp needle to release the pollen mother cells (PMCs). The anther wall and other debris are carefully removed with the needle. At this stage, more stain may be added if needed. A drop of Hoyer's solution or another suitable clearing medium can be added to flatten the cells and clear the background. A clean coverslip is lowered gently over the preparation and pressed firmly first at the

corners and then along the edges to spread the cells into a thin monolayer. This pressure gives the technique its name - the squash. To improve visibility, the slide may be briefly passed over a flame, then pressed under several folds of blotting paper to remove excess stain and prevent air bubbles. If overstaining occurs, the preparation can be destained by carefully washing with dilute acetic acid or distilled water. The slide is now ready for microscopic observation, and well-spread PMCs at diakinesis or metaphase provide the clearest chromosome counts.

5) Destaining of PMC smears

Destaining is done by gently flooding the cover slips on the slide with 45 per cent acetic acid and then keeping the slides in an acetic acid chamber for one to two hours. After destaining, uniform pressure is applied on the cover slip, keeping it in between the several folds of blotting paper to remove excess stain and for good pressing of PMCs

6) Examine under the microscope:

Once the slide is prepared, scan it at low power to find well-flattened cells. The edges of the coverslip often have the best, fully spread

cells. Switch to high power (100X oil immersion) to clearly see the chromosomes. Count the brightly-stained rods or X-shapes in each cell. Look for cells in late prophase or metaphase of meiosis I. At this stage, homologous chromosomes are paired and maximally condensed, so you see n pairs (where n is the haploid number). Each such cell tells you the chromosome number of that species. Successfully counting one sample often requires a few tries (to find the right bud stage), but once you know the correct bud size, repeat counts are easy.

7) Recording of observations

After preparing the slides with proper staining and observing them under the microscope, the meiocytes are scanned for various stages. For determining the chromosome number, the diakinesis or Metaphase I stages are important for selecting various associations; univalents, bivalents, quadrivalents, and other multivalent forms can be counted, and the frequency of different chromosome configurations is recorded. Meiocytes can also be observed at Anaphase I and Anaphase II for

chromosome movement and for chromosome abnormalities, such as chromosome bridges and lagging, arising from incomplete separation. Meiocytes are observed at the Telophase II and tetrad stages for abnormalities such as micronuclei formation. Reading the prepared slide is usually the best part. Meiosis involves several stages during which chromosomes are counted. The easiest is diakinesis (very late prophase I) when each pair of homologous chromosomes is fully aligned. At diakinesis, you will see tight X or circular paired structures, usually well-separated from one another. Each of those is a chromosome pair, so counting them gives the haploid number n . Metaphase I - just after diakinesis is also good, although the pairs are slightly closer together. In later stages, the pairs split, with anaphase I showing two halves. In practice, one can scan the slide and note any stage visible, then calculate n accordingly.

Conclusion

Chromosome squashes are a classic, low-tech window into plant genomes. This century-old technique lets anyone with a

microscope see the chromosomes themselves, the ultimate proof of polyploidy or genome change. Today's botanists are rediscovering its power and economy. This method reveals an aspect of the plant genome accessible with only the most basic resources. Mastering the squash, researchers worldwide can, even with minimal lab funds, produce new chromosome counts for hundreds of unstudied species.

References:

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