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PROTOCOL NOTE

Unfurling an improved method for visualizing mitotic chromosomes in ferns

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Abstract

Premise: Cytotaxonomy employs chromosome visualization to study organismal relationships and evolution. Despite the critical value of cytogenetic data, cytotypes are lacking for many plant groups. Here, we present an improved approach for visualizing mitotic chromosomes in ferns, a key lineage of land plants, using the dividing cells of unfurling croziers (fiddleheads).

Methods and Results: Our modified mitotic chromosome preparation incorporates a brief pectinase–cellulase pretreatment, as well as colchicine fixation and the Feulgen reaction to improve the staining and separation of mitotic chromosomes. To demonstrate this easy and efficient assessment, we determined the sporophytic ($2n$) chromosome number for three fern species: *Cheilanthes mollis* ($2n = 60$), *Cheilanthes hypoleuca* ($2n = 120$), and *Nephrolepis cordifolia* ($2n = 82$).

Conclusions: The new method presented here improves visualizations of mitotic chromosomes from the dividing nuclei of young fern croziers. Fiddleheads are widely accessible in nature and in living collections worldwide, and this modified approach increases their suitability for fern cytotaxonomic studies.

KEYWORDS

Cheilanthes hypoleuca, *Cheilanthes mollis*, Cheilanthoideae, cytology, karyotype, Nephrolepidaceae, *Nephrolepis cordifolia*, whole-genome duplication

The chromosome number of a species is crucial for understanding biological organization (Guerra, 2008; Mayrose and Lysak, 2021). By visualizing chromosomes, researchers can shed light on natural variation in size, shape, and number that reflects key evolutionary processes, such as polyploidization (i.e., whole-genome duplication), within a lineage (e.g., Mayr, 1963; Clark and Donoghue, 2018; Hörandl, 2022). Examining chromosome copy number (i.e., cytotype) can yield indispensable insights into species relationships, the speciation process (e.g., Jones and Ruban, 2019; Maragheh et al., 2019; Lysak and Weiss-Schneeweiss, 2021), and the profound impacts of polyploidization on genome organization and function (Bennett and Leitch, 2001; Wang et al., 2022).

Cytotype is especially critical for studies involving taxa that are prone to whole-genome duplication, such as ferns (Wood et al., 2009; Zhang et al., 2019; Pelosi et al., 2022). Despite the immense value of cytogenetic evidence, verifiable reports of chromosome number remain limited, even in relatively well-studied groups like ferns that are characterized by variation in base chromosome number (x) and frequency of polyploidy (Barker et al., 2016; Hanušová et al., 2019; Liu et al., 2019; Barrington, 2020; Wang et al., 2022). Gaps in cytotype data present major impediments when addressing fundamental biological questions and when accurately assembling organismal genomes (Araya-Jaime et al., 2021), even if complementary approaches, such as flow cytometry or ecological assessments, are employed (Levin, 2002; Clark et al., 2016; Windham et al., 2020).

The chromosome squash technique is the most widely used and reliable method for studying mitotic or meiotic chromosomes in plants and is notable for producing accurate

estimates of both chromosome number and ploidy level (Kirov et al., 2014; Nakato and Ebihara, 2016; Singh, 2016; Windham et al., 2020). In plants, all chromosome squash methodologies involve three essential steps: tissue collection, fixation, and staining (Ebihara et al., 2014; Sharma and Sharma, 2014; Singh, 2016; Windham et al., 2020).

In the first step, meristematic or germ cell tissues are collected from growing sporophytes (or gametophytes) that are actively undergoing cell division (Kirov et al., 2014; Singh, 2016). For observations of mitosis, sporophyte ($2n$) root tips are traditionally used because they bear a root apical meristem (Mandáková and Lysak, 2016; Aragón-Raygoza et al., 2020). In many ferns, however, root cells are comparatively few and their thick epidermal cell walls and root cap tissues can hinder diffusion and permeability during fixation, resulting in decreased potency of mitotic inhibitors (personal observation; Motte et al., 2020). To avoid challenges associated with mitosis, meiosis can serve as an alternative. In ferns, meiosis takes place within developing sporangia on young sporophyte leaves (Haufler et al., 2016). However, as with angiosperms, the timing of meiosis in ferns can be difficult to predict (Yang et al., 2022), limiting access to meiotic tissues for studies that involve wild or remote populations or that occur in a laboratory setting, where newly developing fern sporophytes can take years to achieve reproductive maturity (Manton, 1950; Britton, 1953; Ye et al., 2007; Singh, 2016).

Next, freshly sampled tissues are usually submerged within a mitotic inhibitor and a fixative that causes dividing cells to arrest at metaphase (Johansen, 1940; Sharma and Sharma, 2014; Singh, 2016). Selecting an appropriate fixative is a critical feature of chromosome squashing protocols that can drastically impact the accuracy of resulting observations (Huang and Yeung, 2015; Singh, 2016). In addition to stopping cell division, fixatives also preserve cellular and chromosomal morphology, preventing changes from occurring during subsequent staining and preparation steps (Johansen, 1940; Singh, 2016). Suboptimal fixation can result in distorted chromosomes or altered cellular structures, leading to inaccurate counts and impacting downstream analyses (Dietrich, 1986; Sharma and Sharma, 2014; Singh, 2016). Furthermore, the choice of a suitable fixative can be crucial for overall preparation quality (Johansen, 1940; Singh, 2016; Windham et al., 2020). For instance, fixatives like formalin–acetic acid–alcohol (FAA) are used for anatomical or morphological studies in some plant species but are inappropriate for the preservation of small chromosomes (Johansen, 1940; Setiawan et al., 2018), such as those typically found in ferns (Clark et al., 2016), for which a mixture of absolute ethanol and acetic acid in a proportion of 3:1, called Farmer's fixative, is used (Setiawan et al., 2018; Windham et al., 2020).

After fixation, stain is applied to increase the visibility of DNA within the cell nuclei. The Feulgen reaction is among the most commonly utilized methods for nucleic acid staining (Navarrete et al., 1983; Mello and Vidal, 2017). Developed more than 100 years ago (Feulgen and Rossenbeck, 1924; Navarrete et al., 1983; Chieco and Derenzini, 1999), the Feulgen reaction is a histochemical technique for detecting DNA in chromosomal studies that involves hydrolysis and a colored reaction that is specific to DNA and, thus, chromosomes (Darlington and La Cour, 1960; Chieco and Derenzini, 1999; Mello and Vidal, 2017). Compared to other stains, such as acetocarmine and orcein, the Feulgen reaction is more precise in its ability to identify DNA (Hardie et al., 2002; Mello and Vidal, 2017). The Feulgen reaction is based on the binding of Schiff's reagent to DNA and serves as a permanent staining technique that allows for the easy detection of DNA, even in small amounts (Chieco and Derenzini, 1999; Singh, 2016; Mello and Vidal, 2017; Itgen et al., 2022). The result is a purple-red staining of the DNA that can be easily visualized under any light microscope with sufficient magnification (Navarrete et al., 1983; Mello and Vidal, 2017; Itgen et al., 2022).

As previously mentioned, the most common method for visualizing mitotic chromosomes in plants involves preparing a squash of cells derived from the root apical meristem (Shinohara et al., 2010; Singh, 2016; Windham et al., 2020; Kono et al., 2022). In ferns, however, root meristematic tissues are minimally abundant, leading to low effectiveness of mitotic spindle inhibitor treatments (personal observation; Harrison and Morris, 2018) and making the use of conventional cytogenetic protocols difficult. Obtaining well-spread mitotic karyotypes from ferns is further complicated by physical constraints, primarily owing to the presence of cell walls (Mandáková and Lysak, 2016) and the absence of sufficiently detailed methodologies (Kirov et al., 2014; Windham et al., 2020).

To overcome these difficulties, we developed an alternative approach, designed specifically for ferns, that uses tissue from unfurling croziers (fiddleheads), instead of roots. To improve upon existing protocols (e.g., Limpanasittichai and Jaruwattanaphan, 2022), we provide a detailed method that incorporates a brief cellulase–pectinase pretreatment before the mitotic spindle treatment and employs the Feulgen reaction to facilitate the visualization and promote the separation of mitotic chromosomes (Feulgen and Rossenbeck, 1924; Navarrete et al., 1983; Chieco and Derenzini, 1999; Mello and Vidal, 2017). The method presented here expands the available toolkit for studying cytotype variation in ferns, a major clade of land plants with a proclivity for whole-genome duplication (Barker and Wolf, 2010; Huang et al., 2020; Kinosian et al., 2022). Additionally, given the critical phylogenetic position of ferns as the sister lineage to seed plants (Pryer et al., 2001; Wood et al., 2009; Kinosian et al., 2022), this improved method has the potential to yield broad impacts for plant systematics and evolution.

METHODS AND RESULTS

We provide a detailed list of supplies and reagents, as well as a basic troubleshooting guide and a summary of our protocol, in Appendix 1, to supplement the methods described below.

Tissue collection and sampling

We sampled unfurling croziers from sporophytes (2*n*) of *Cheilanthes hypoleuca* (Kunze) Mett. (Pteridaceae), *Cheilanthes mollis* (Kunze) C. Presl (Pteridaceae), and *Nephrolepis cordifolia* (L.) C. Presl (Nephrolepidaceae) growing at the Universidad de la Serena greenhouse in La Serena, Chile, for this study. Voucher specimens were prepared for each plant sampled, and specimens were deposited in the herbarium at the Universidad de la Serena (ULS; see Appendix S1).

First, meristematic regions were removed from 2–5 developing croziers per individual, each crozier approaching a height of 1–1.5 cm measuring from the base of the petiole. Preferential sampling was given to younger fronds that appeared relatively thin and slightly lighter green among croziers of appropriate height (Figure 1), rather than older unfurling fiddleheads. A scalpel (or sharp scissors) was used to carefully cut each crozier near the base of the petiole. Once collected, croziers were briefly stored in a damp paper towel or plastic bag at room temperature before applying the enzyme pretreatment.

Enzyme pretreatment

To improve permeability of the vegetative tissue and to obtain better results with the downstream spindle inhibitor treatment, croziers were subjected to a lysing enzyme pretreatment. First, the croziers were washed 2–3 times in KC buffer (pH 4.3) for at least 5 min (Appendix 1). After washing, croziers were immersed in 5 mL of lysing enzyme (2.5% cellulase–pectinase in KC buffer) and incubated for 5–10 min at 40°C, using a shaker (DLAB-HM100 Pro; DLAB Scientific, Riverside, California, USA) to promote efficient mixing. Once the incubation was complete, the lysing enzyme was removed by washing the vegetative tissues with a mixture of distilled water and tap water at least three times. Washing with water helped to keep the cells alive and to remove any residual lysing enzyme that could interfere with subsequent steps of the protocol. After washing, the vegetative material was ready for exposure to the spindle inhibitor treatment.

FIGURE 1 Young fiddleheads (croziers). (A) Young croziers growing in a greenhouse environment. (B) Young croziers of *Nephrolepis cordifolia*, *Cheilanthes mollis*, and *C. hypoleuca* (left to right) sampled for cytogenetic analysis.

Mitotic spindle inhibitor treatment

Treating the vegetative material with diluted colchicine (0.05%) was crucial for obtaining contracted chromosomes that were easy to count (Figure 2) and for increasing the number of metaphase chromosome plates without spindle fibers (Dhooghe et al., 2011; Singh, 2016). Colchicine disrupts the formation of microtubules, leading to the arrest of cell division at metaphase. During this time, chromosomes become separated and distributed randomly within the cell space (Singh, 2016). The treatment was carried out for 3–4 h at room temperature (18°C), allowing sufficient time for the colchicine to take effect, and thereby facilitating the easy visualization of mitotic chromosomes. In some cases, an adjustment of 30 min, either reducing or extending the duration of the colchicine treatment, was needed (Figure 2D, E). Following the colchicine spindle inhibitor treatment, vegetative material was washed three times with tap water to remove residual colchicine.

Tissue fixation

To remove pigmented compounds, such as chlorophyll, from the croziers and to preserve the cells in a specific stage of cell division without causing shrinkage, distortion, or bacterial growth, we used Farmer's fixative, which is prepared by mixing 95% ethanol and glacial acetic acid in a 3:1 ratio (Johansen, 1940; Setiawan et al., 2018; Windham et al., 2020). We immersed the croziers in this solution for a minimum of 12 h. Notably, Farmer's solution was freshly prepared, immediately prior to fixation with colchicine. To prevent undesirable hardening of the tissues, the material was not stored for a prolonged period (<24 h) after immersion into the fixative. Material was then transferred out of the fixative and into 70% ethanol for long-term storage, where it can be preserved for up to one year at 18°C (possibly longer if stored at −20°C) (personal observation).

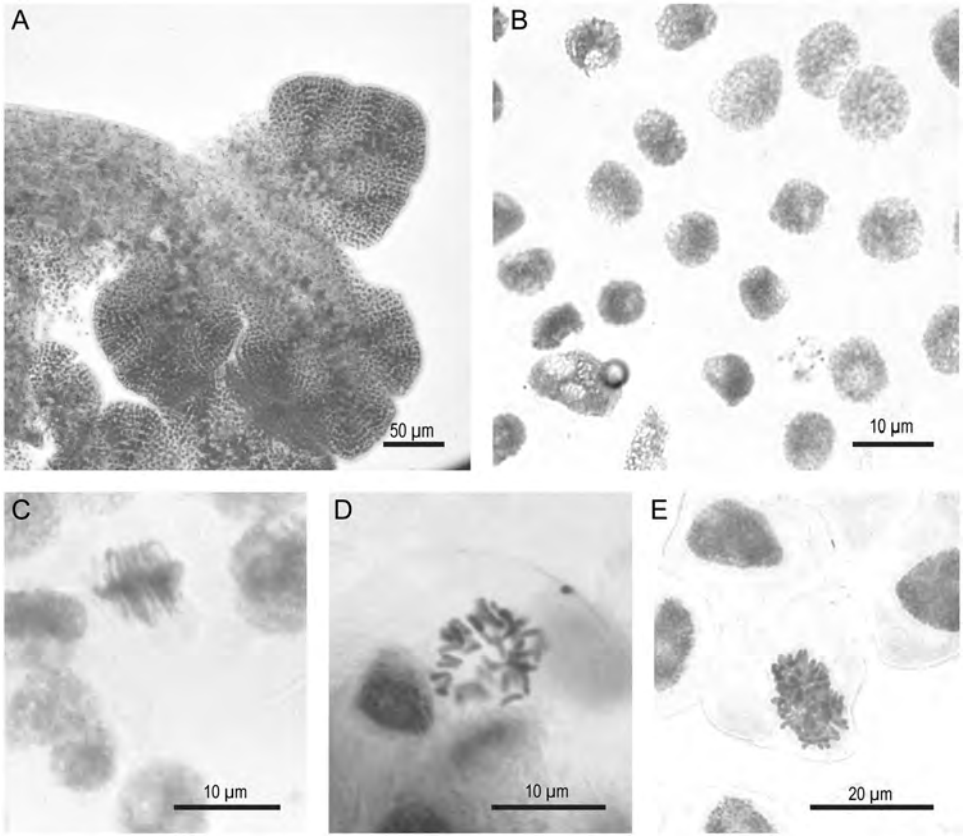

FIGURE 2 Cells from an unfurled crozier of *Cheilanthes hypoleuca* (A–D) and *C. mollis* (E) stained using the Feulgen reaction. (A) An apical segment of the unrolled crozier. (B) Cells released under the second, post-Feulgen enzyme treatment. (C) Cells undergoing mitosis without the effect of the spindle inhibitor treatment. (D) Cells undergoing mitosis with optimal spindle inhibitor treatment time. (E) Cells undergoing mitosis with excessive time in the spindle inhibitor treatment.

Nucleic acid staining

The Feulgen reaction is a common staining approach that can be used to determine the amount of DNA within cells through image cytometry (Feulgen and Rossenbeck, 1924; Chieco and Derenzini, 1999). The Feulgen reaction has two steps: HCl hydrolysis to produce free aldehyde groups and a subsequent color reaction that is caused by exposing cells to Schiff's reagent (Feulgen and Rossenbeck, 1924; Navarrete et al., 1983; Chieco and Derenzini, 1999). To perform the Feulgen reaction on fixed material, we first rehydrated the croziers by washing them 2–3 times in distilled water. We then hydrolyzed them by submerging in 5 N HCl (in 1.5-mL glass vials or 10-mL glass bottles) for at least 2 h. After hydrolysis, croziers were washed with distilled water three times for approximately 10 min. Excess water was removed and the Schiff's reagent was added (Appendix 1) and allowed to react for a minimum of 2 h or until a distinct magenta color emerged (Figure 2).

Sulfur water bath

To remove excess Schiff's reagent, we prepared 60 mL of sulfurous water by mixing 5 mL of 10% sodium metabisulfite and 5 mL of 1 N HCl in 50 mL of distilled water and washed the material three times for at least 5 min each.

Softening and squashing croziers

To ensure a clean and well-spread preparation, we then treated the fixed material with a freshly prepared 5% cellulase–pectinase enzyme solution (dissolved in citrate buffer at pH 4.7; Appendix 1) for a minimum of 2 h at 37°C. This enzymatic treatment softened the tissue and facilitated the release of chromosomes during the subsequent squashing step. After the cellulase–pectinase treatment, the material was washed with a citrate buffer (Appendix 1) to remove any remaining reagents.

Unfurling the crozier

In preparation for squashing, soft meristematic tissues with a high mitotic index, distinguishable by magenta-stained chromosomes, are needed (Figure 2D). To this end, the fixed fiddleheads were unfurled in 50% glacial acetic acid in

a glass Petri dish using a dissecting needle and precision tweezers (electrostatic discharge [ESD] No. 11-12) and observed using a stereoscopic microscope (Motic SMZ 140; Motic, Hong Kong, China). For samples bearing leaf scales, the scales were carefully removed (Figure 2A).

Conducting the squash

For best results and easy counting, microscope slides were cleaned thoroughly, submerged entirely into 70% ethanol, and then generously polished on both sides with a KimWipe (Kimberly-Clark, Irving, Texas, USA) until shiny and clean. Note that, if long-term preservation is desired, gelatinized slides can be used for this step; detailed instructions on how to make and use gelatinized slides are provided below and in Appendix 1. Next, the innermost segment of the unfurled crozier (Figure 2A) was placed on the slide along with 1–2 drops of 50% glacial acetic acid and a coverslip (Hirschmann Laborgeräte, Eberstadt, Germany), gently applied, ensuring that the amount of 50% glacial acetic acid was sufficient to create an entire liquid layer between the coverslip and the slide. Forceps (or a probe) were used to gently tap the coverslip and rupture the meristematic tissue, releasing the cells (Figure 2B). The resulting samples were scanned at 400× magnification to identify cells suitable for analysis (Figure 3), specifically focusing on chromosome spreads devoid of spindle fibers (Figure 2D). After identifying the most promising regions (Figure 2D), gentle thumb pressure was applied to achieve a single layer of fully flattened cells. Chromosome spreads from *Cheilanthes hypoleuca*, *Cheilanthes mollis*, and *Nephrolepis cordifolia* were captured using an optical microscope (Nikon Eclipse E-400; Nikon, Tokyo, Japan) equipped with a Nikon 10-MP CMOS camera, operating at a magnification of 1000× and utilizing immersion oil to enhance image quality (Figure 4).

Preparation of permanent slides

For the preceding "squashing" step, we used gelatin-coated slides (Appendix 1). Following image capture, we labeled each slide and transferred them to a −80°C freezer (or dry ice) overnight to ensure complete freezing of the slide to preserve the cellular structures and prevent subsequent changes.

Once frozen, slides were removed from the cold and the coverslip was carefully removed by lifting at the corners with a scalpel. Caution was taken to avoid disturbing or damaging the cells on each slide. Slides were then placed on a hot plate (ca. 60°C) or in a warm, dry area and allowed to dry thoroughly, ensuring all water droplets had evaporated. This step was essential to prevent water residue from accumulating that might interfere with the final mounting of the coverslip.

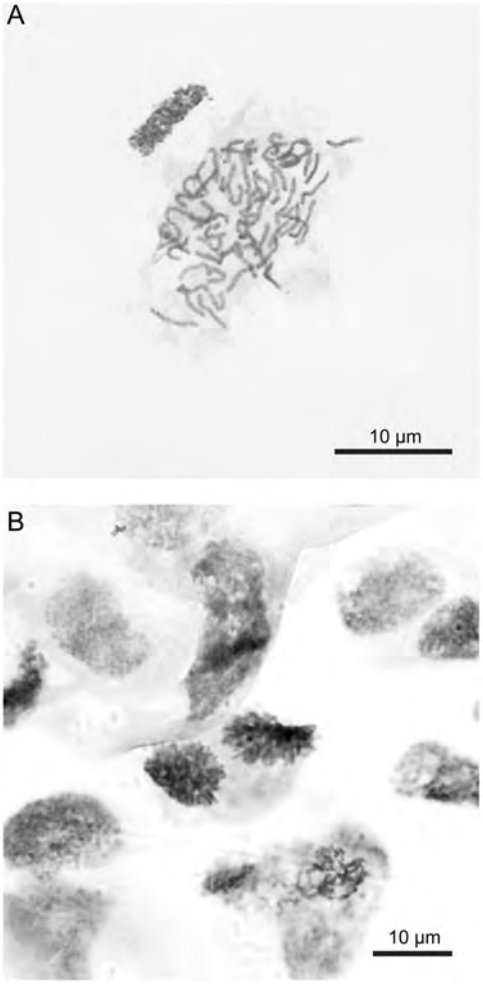

FIGURE 3 Additional stages of the cell cycle in *Cheilanthes hypoleuca* observed using the mitotic chromosome protocol. (A) Early prophase. (B) Late anaphase.

Next, slides were submerged in a Coplin jar filled with xylitol (Appendix 1), submerging the cells of interest while keeping the slide label above the surface for 10 min. Precisely two drops of mounting medium (Appendix 1) were then placed onto a coverslip. The slide was carefully removed from the xylitol to ensure that the side with cells was facing downward, towards the coverslip with mounting medium. The slide was placed onto the coverslip, with proper contact between the slide and coverslip, by gently pressing down on the slide with a thumb to eliminate any trapped air bubbles. Then, the slide was placed upright, and gentle pressure was applied to the coverslip while checking for any remaining air bubbles, as their presence can interfere with later examination of the specimen. To ensure that no air bubbles were present between the slide and the coverslip, and that the slide was completely dry, the slide was allowed to sit for 1–2 h at room temperature. Finally, a permanent label was applied to the slide, and it was stored in a slide box for downstream examinations and archival purposes.

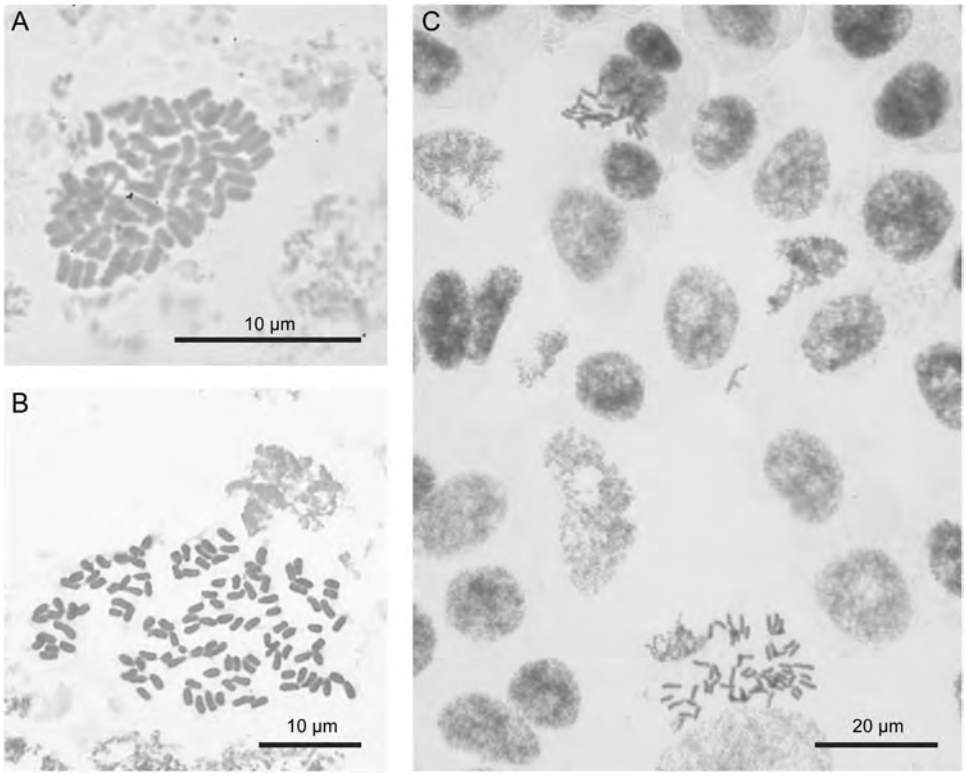

FIGURE 4 Chromosome spreads from three fern species stained using the Feulgen reaction. (A) *Cheilanthes hypoleuca* ($2n = 60$). (B) *Cheilanthes mollis* ($2n = 120$). (C) *Nephrolepis cordifolia* ($2n = 82$).

CONCLUSIONS

Our findings align with the conclusions drawn by Windham et al. (2020) regarding the value of chromosome squashes as a cost-effective and efficient method for studying chromosome numbers and their morphology. This technique serves as a critical tool for researchers seeking to investigate variation in chromosome size, shape, and number, which is pivotal for understanding key evolutionary processes, such as polyploidization, within lineages. In particular, the study of chromosome and genome copy numbers remains crucial to unraveling the complexities of plant evolution, especially in reticulate polyploid complexes.

Our novel approach for visualizing mitotic chromosomes in ferns addresses several challenges that are encountered when applying traditional protocols designed for angiosperms. By utilizing fern croziers (fiddleheads) as an alternative source of mitotic material, and by incorporating an enzyme pretreatment as a preliminary step in the chromosome preparation workflow, we improve upon existing methods. By leveraging the Feulgen reaction, we enhanced the separation and staining of mitotic chromosomes. Notably, our extended cellulase–pectinase enzyme treatment resulted in well-dispersed and visually pleasing chromosome spreads, enabling accurate determination of chromosome numbers for *Nephrolepis* and *Cheilanthes* species (*C. mollis*, $2n = 60$; *C. hypoleuca*, $2n = 120$; and *N. cordifolia,* $2n = 82$).

AUTHOR CONTRIBUTIONS

R.R.C. coordinated the project. A.L.G., R.R.C., C.A.J., P.J.S., and C.P.R. designed the project. R.R.C., C.P.R., and C.A.J. fine-tuned the protocol and confirmed reproducibility in the lab. R.R.C. and C.P.R. obtained microscopy images. R.R.C., C.P.R., P.J.S., A.L.G., and C.A.J. wrote and revised the manuscript. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

Chromosome preparations are provided within this manuscript as high-resolution figures. A list of necessary chemicals and equipment, suggestions for troubleshooting, and a brief summary of the protocol are provided in Appendix 1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Sample and voucher information for specimens used in this study for chromosome number determination.

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Appendix 1. Detailed protocol and comprehensive list of essential materials and reagents.

Chemicals and solutions

99% ethanol or absolute alcohol
Glacial acetic acid
Schiff's fuchsin–sulfite reagent
Entellan mounting medium
Immersion oil
Cellulose from *Aspergillus niger*
Pectinase from *Aspergillus niger* in powder
Gelatin (USP grade)
Sodium metabisulfite at 97%
Distilled water
Giemsa stain
Calcium chloride
Colchicine
Potassium chloride
HCl, 37%
Basic fuchsin (C.I. 42500)
Activated carbon (USP grade)
Thymol crystal
Chrome alum

Supplies

Glass Petri dish
Dissecting scalpel
Vials (1.5 mL) (with pressure caps)
Glass bottles (5–10 mL) (with spill-proof safety caps)
Microscope slides (75 × 25 mm)
Microscope slide coverslips (16 × 16 mm) (Hirschmann Laborgeräte, Eberstadt, Germany)
Slide racks
Staining dish
Beaker (1000 mL)

Equipment

Electronic precision tweezers (No. 11-12)
−80°C freezer
Hot plate with magnetic stirrer (No. 78624; Torrey Pines Scientific, Carlsbad, California, USA)
Dissecting microscope (Motic SMZ-140; Motic, Hong Kong, China)
Optical microscope with camera (Nikon Eclipse E-400 with a mounted Nikon 10-MP CMOS camera; Nikon, Tokyo, Japan)

Budget-friendly recipes

Schiff's reagent

1 g basic fuchsin
200 mL distilled water
10 mL 1 N HCl
2 g sodium metabisulfite
200 mL distilled water
1.5 g activated carbon

Dissolve the basic fuchsin in 200 mL of distilled water and then add the metabisulfite and 10 mL of 1 N HCl. Shake the mixture vigorously for 2 h. Once completely dissolved, allow the solution to rest undisturbed in a light-free environment for 48 to 72 h. After this incubation period, filter the solution with 1.4 g active carbon. The resulting filtrate should exhibit a colorless appearance. Finally, store the filtered solution at a temperature of 5°C for extended periods of up to 6 months.

KC buffer
400 mL distilled water
17.9 g potassium chloride
2.95 g calcium chloride dihydrate

Dissolve the potassium chloride in distilled water at room temperature using a magnetic stirrer, then add the calcium chloride until it is completely dissolved. Store at 2–8°C until needed.

Citrate buffer (pH 4.2)
1000 mL distilled water
25.703 g sodium citrate dihydrate (0.2 mol)
2.421 g citric acid (0.2 mol)

Prepare the solution by dissolving sodium citrate in 800 mL of water, then add the citric acid. Adjust the pH of the solution to the desired level using a pH meter. Finally, add the remaining water gradually until the total volume reaches 1000 mL.

Gelatin-coated slides
5 g gelatin
5 g chrome alum
1000 mL distilled water
1 crystal thymol

To prepare the gelatin solution, dissolve gelatin in 1000 mL distilled water at room temperature using a magnetic stirrer. Once the gelatin is completely dissolved, add 5 g chrome alum and one thymol crystal; continue stirring until fully dissolved. Store the solution at 2–8°C.

For the final preparation of the gelatin-coated slides, place clean histological slides into a slide rack and position the rack inside the staining dish. Heat the gelatin solution to 40°C and carefully pour it into the staining dish until the slides are completely submerged. Remove the slide rack from the solution and allow the slides to dry.

Frequently encountered challenges and possible solutions

Too much mitotic inhibitor effect or no mitotic inhibitor effect: If you observe chromosome agglutination in the mitotic spreads, reduce the mitotic inhibitor treatment by 30 min. Conversely, if your samples yield metaphase spreads without the mitotic inhibitor effect (Figure 2C), extend the colchicine treatment for another 30 min.

Scales and trichomes prevent complete flattening during the squashing step: In many fern species, newly developing croziers are often accompanied by abundant scales or trichomes. To ensure a complete cellular flattening without interference from these scales or trichomes, an additional step is needed following the application of sulfur water to remove excess Schiff's reagent. Using a dissecting microscope and precision tweezers, carefully remove the outermost layer of young leaves that constitute the crozier along with their associated trichomes and scales. If any indument remains visible, continue removing but leave other structures undisturbed. The innermost layers will then be a suitable region for squashing.

The chromosomes are poorly stained: After storing samples in the −80°C freezer, it is common to observe a slight discoloration of the chromosomes. Therefore, we recommend immersing the slide in Giemsa stain for a minimum of 3 min. Then, proceed with the workflow to create a permanent slide.

Step-by-step protocol for squashing chromosomes using fern croziers

1) Collect young croziers from a living plant from the field or a greenhouse.
2) Briefly submerge the crozier in a cellulose–pectinase solution diluted to 2.5% in KC buffer for 5 min.
3) Remove the crozier from the enzyme pretreatment and submerge it in a diluted colchicine solution (0.05%) for a minimum of 3 h to inhibit mitotic division.
4) Immerse the treated croziers in Farmer's fixative (a 3:1 ratio of alcohol to acetic acid) for at least 12 h.
5) After rehydrating the fixed material, perform the nucleic acid staining by submerging the crozier in 5 N HCl for 2 h. Thoroughly wash the crozier three times with distilled water and then add Schiff's reagent. Allow the material to remain in the Schiff's reagent until a magenta color develops.
6) Once excess stain has been removed using a sulfurous water bath, proceed with the long enzymatic softening treatment. Submerge the crozier in a 10-mL glass vial containing 5 mL of cellulose–pectinase solution at 5% concentration. Allow the crozier to remain in the solution for 2 h or until the material becomes sufficiently soft.
7) Extract a piece of the innermost layer of leaves with bright magenta cells and place it onto a clean slide (see steps 9 and 10, below) to perform the chromosome squash.
8) For the chromosome squash, add two drops of 50% glacial acetic acid, then carefully place a coverslip on top of the tissue. Using a dissecting needle, tap the coverslip where the tissue is located to rupture and disperse the cells. Once the cells are ruptured, locate the most suitable region for observation of chromosomes and press on this area gently with your thumb. At this point,

you will be able to observe flattened chromosome spreads.

For long-term, permanent storage:

9) Instead of using a recently cleaned slide in step 7, use a gelatin-coated slide. Follow the workflow until step 8. Label the prepared slide and transfer it into a −80°C freezer (or place it on dry ice) for freezing overnight. After freezing, carefully remove the coverslip from the corner using a scalpel. Place the slide on a hot plate to ensure complete evaporation of any remaining water droplets.
10) Next, submerge the slide into a Coplin jar filled with xylitol. Place two drops of mounting medium onto the coverslip. Remove the slide from the jar, then position it over the coverslip and press until all the air bubbles are gone. Finally, flip the permanent slide preparation upright and let it dry.