CYTOGENETIC TOOLS TO INVESTIGATE THE COMPILOSPECIES

by

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Abstract

A compilospecies is defined as a genetically aggressive taxa that "steals" or incorporates the genomes of other taxa via introgressive hybridization. This concept was first defined in *Bothriochloa, Capillipedium*, and *Dichanthium* (the BCD clade). Within this clade, *Bothriochloa bladhii* was identified as the compilospecies. Proper cytological techniques and accurate chromosome counts are needed to begin investigations to understand the mode of hybridization and to track "dominant" genomes. Techniques for preparation of meiotic chromosome spreads in species with more than 60 chromosomes are lacking in the literature. In this study, two methods for preparing chromosomes in the BCD clade are presented. These methods are adapted from the 'SteamDrop' (Kirov et al., 2014) and modified drop method (Kuo et al., 2016). Recommendations for further cytological work on the BCD clade based on these experiences are also suggested.

Introduction

Jack Harlan and J.M.J. de Wet (1963) described a new species concept that depicts how polyploidy complexes evolve through a mechanism of introgressive hybridization and genome dominance. They termed this the compilospecies concept. The combination of hybridization and genome dominance results in a large range of morphological variation within species and across genera, in some cases (R. P. Celarier & Harlan, 1955). For this reason, the compilospecies concept cannot be studied through morphological or geographical data. In 2014, Estep et. al. built a phylogenetic tree to study the compilospecies in bluestem grasses (*Bothriochloa, Capillipedium,* and *Dichanthium*), the same system Harlan and de Wet used to describe the compilospecies concept. The phylogenetic analysis verified the polyploidy complex and that gene flow between members of the BCD clade had occurred, but concluded it was impossible to study this concept using phylogenetic data alone. The large range of morphological variation paired with high rates of introgression in the BCD clade make it difficult to study the compilospecies concept using morphological, geographical, or phylogenetic data alone. Development of modern cytological tools are needed to study chromosome structures, number, and to further explore this concept. In this study, a cytological method to prepare chromosomes in *Bothriochloa, Capillipedium*, and *Dichanthium* (BCD clade) is presented.

The Compilospecies Concept

A compilospecies is defined as a genetically aggressive taxa that “steals” or incorporates the genomes of other taxa via introgressive hybridization (Jack R. Harlan & de Wet, 1963).

Introgressive hybridization is the gradual introduction of germplasm of one species to another as a result of hybridization and repeated backcrossing (Figure 1A). When a compilospecies undergoes a hybrid cross, the compilospecies' subgenome exhibits genome dominance over the second parents subgenome. Genome dominance can be thought of as similar to allelic dominance, but on a larger scale. As a result, the daughter taxon phenotypically resembles the compilospecies (Figure 1B). The daughter hybrid then backcrosses into each parental population introducing new genetic material. Here, genetic material from the dominant genome is gradually introduced to the recessive genome. The introduced dominant genome material is expressed preferentially over the recessive genome causing the recessive population to start showing phenotypic traits of the dominant species. Introgression events over time may result in the dominant genome, the compilospecies, fully absorbing another species via ass imilation of genetic material.

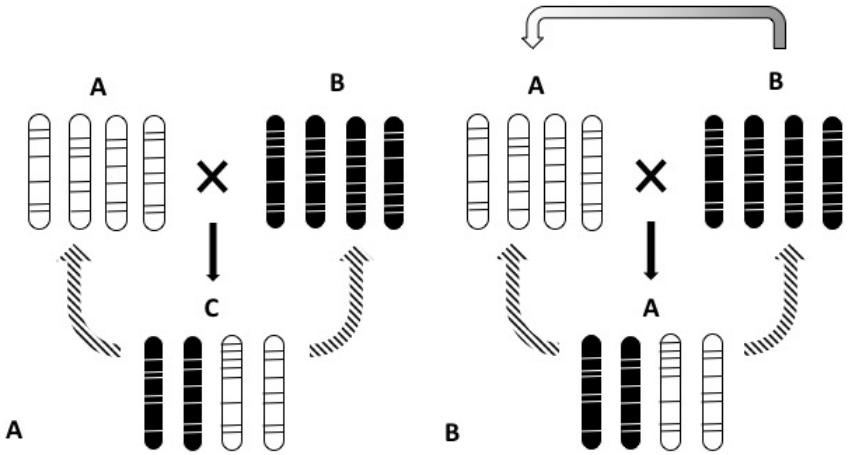

Figure 1. Modes of hybridization. Letters indicate the species that the genome looks like morphologically. White bars are chromosomes of species A and black bars are chromosomes of species B. Dashed arrows are backcrossing. A gradient arrow indicates introgression over time. A) Introgressive hybridization. A and B cross to produce a morphologically unique hybrid. This hybrid backcrosses into the parental populations. B) The compilospecies concept. A and B cross to produce a hybrid that morphologically resembles A. Introgression of the hybrid into the parental populations over time results in species B phenotypically resembling species A.

Jack Harlan and J. M. J. de Wet (1963) first defined the compilospecies concept in *Bothriochloa* Kuntze, *Capillipedium* Stapf, and *Dichanthium* Willemet (the BCD clade). Commonly known as the bluestem grasses, the BCD clade is globally distributed in temperate and tropical grasslands (GBIF *Bothriochloa*, 2017; GBIF *Capillipedium* 2017; GBIF *Dichanthium* 2017). The three genera have distinct morphologies that center around a terminal panicle with rames composed of nodes with sessile-pedicellate spikelet pairs (Figure 2). *Bothriochloa* lives up to its common name, beardgrass, with its villous features (Figure 3A). Rames are mostly racemosely arranged and are covered with long hairs (Barkworth, 2003). The lemma contains a vein that extends into a geniculate awn that is bent and twisted and the spikelet nodes are always hairy. *Bothriochloa* also has a distinctive pit in the lower glume on each floret that easily separates it from the other two genera. *Dichanthium* has rames organized in digitate or subdigitate clusters that are basally naked (Figure 3B) (Barkworth, 2003). Spikelets in *Dichanthium* also contain an awn, but it is not geniculate. Lastly, *Capillipedium* has a unique

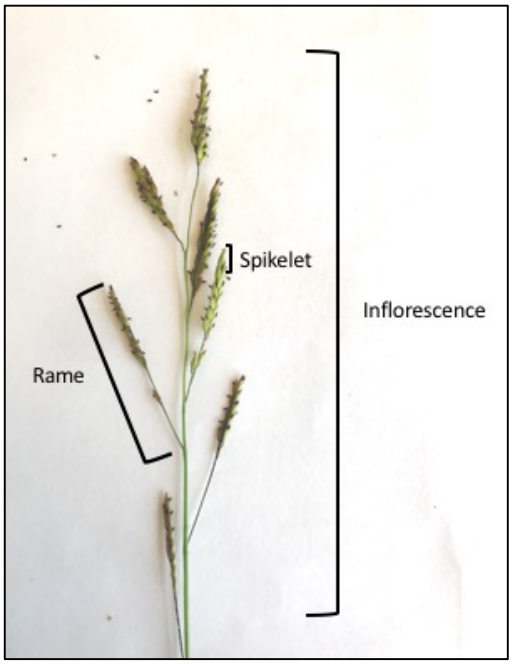

Figure 2. Morphology of a BCD clade inflorescence (*Dichanthium* species). The inflorescence is a terminal panicle. The inflorescence is composed rames. These rames many contain spikelets. Inside of a spikelet is the florest, which contains the anthers.

morphology that appears very open (Figure 3C) (Burbidge, 1984). The rames are racemosely arranged, but have longer internodes and naked margins. There are also fewer spikelets (3-8) on each rame giving the inflorescence a less dense appearance in comparison. *Capillipedium* spikelets also have a geniculate awn, like *Bothriochloa*.

Harlan and de Wet's attention was first brought to this clade when they noticed large morphological variation within species and genera (Figure 4) (R. P. Celarier & Harlan, 1955). This made it challenging to properly identify species and identify the true range of a single species. They noticed that most variation was geographically located in the middle of two well defined species and hypothesized it was a result of gene flow (R. P. Celarier, 1957). Cytological studies were conducted on all variants and artificial hybrids were constructed to understand the genomic features and cytogenetic mechanisms contributing to the plasticity seen across the BCD clade (H. R. Chheda & Harlan, 1962). To do so, they performed aceto-orcein chromosome squashes using anthers fixed in Carnoy's II solution (Borgaonkar, 1960; R. P. Celarier, Wet,

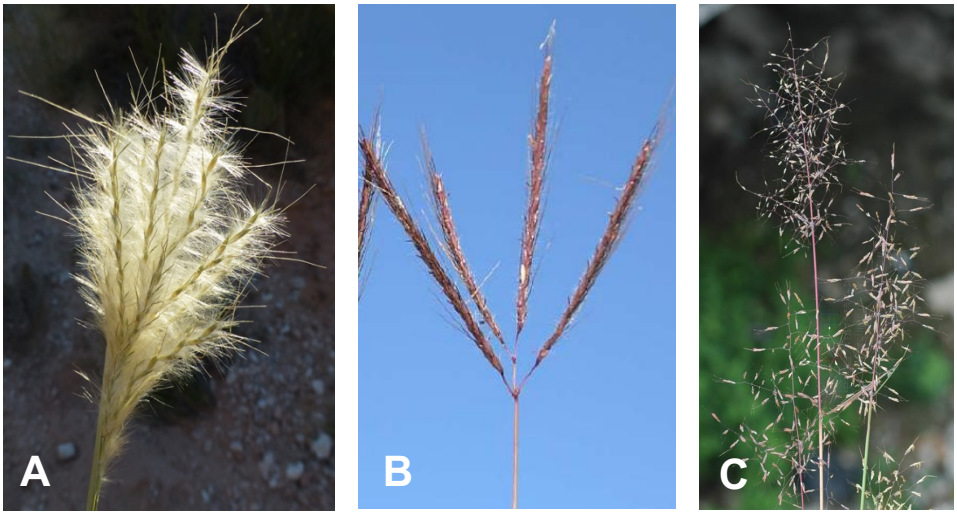

Figure 3. Images of grass inflorescences for the three genera of the BCD clade A) *Bothriochloa* species (spp.) B) *Dichanthium* spp. C) *Capillipedium* spp. (Images provided by Matt Estep).

Borgaonkar, & Harlan, 1961; H. R. Chheda, de Wet, J. M. J., Harlan, J. R, 1961; Wet, Borgaonkar, & Chheda, 1961).

Harlan and de Wet found the BCD clade is a polyploidy complex, meaning it is composed of at least two genetically isolated diploid populations that interbreed through apomictic autopolyploid derivatives (R. P. Celarier, 1957) (Figure 5). Apomixis is an asexual reproduction mechanism often found in polyploidy species where seeds are produced without fertilization (Rieger, Michaelis, & Green, 2012). Facultative apomicts can reproduce sexually via meiosis and asexually via mitosis. Most of the species within the BCD clade are mixed-ploidy species, where small diploid populations overlap in range with more broadly distributed tetraploid and hexaploid populations. The diploids give rise to sexual autotetraploids through

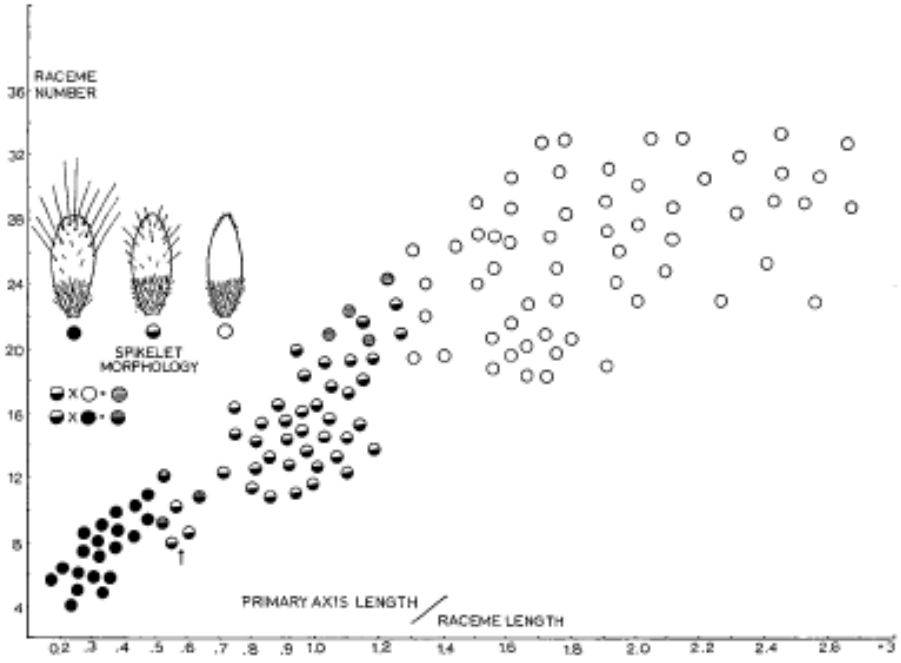

Figure 4. A study in variation of spikelet morphology between *Dichanthium annulatum* (solid circles), *Bothriochloa bladii* (open circles), and artificial hybrids (half-solid, half-open circles). Although the species group individually, defined species boundaries remain unclear because of the large range of variation between species and within a single species. (Jack R. Harlan & de Wet, 1963).

autopolyploidy or hybridization where haploid gametes fuse with apomictic triploids. Sexual autotetraploids can hybridize with facultative apomictic autotetraploids of other species to create genetic bridges between genera. The resultant hybrids are allotetraploid facultative apomicts. The hybrids use apomixis to circumvent the complicated process of chromosome pairing and crossing over that is essential to sexual reproduction. The use of apomixis over sexual reproduction suggests the apomictic mode of reproduction is dominant to sexuality in the BCD clade (de Wet, 1968). Tetraploid hybrids may reduce to haploids through parthenogenetic development of sexual embryo sacs. The haploids behave like diploids cytogenetically and can introgress into the diploid populations. This diploid-tetraploid-haploid cycle allows increased genetic variation, while maintaining species boundaries through ensuring contact with sexuality (Figure 5A) (de Wet & Harlan, 1970).

Hybridization events that occurred between sexual tetraploids have broken down the distinction between species of the BCD clade, not only morphologically but also on a genomic scale. Estep et al. (2014) constructed a phylogeny of the BCD clade and found many species contained multiple copies of the same genetic locus derived from multiple genera. Where the boundaries of these species begin to break down both geographically and phylogenetically, *Bothriochloa bladhii* (Retz.) S. T. Blake is commonly involved (Figure 5B). Harlan and de Wet found hybrids between *B. bladhii* and other members of the BCD clade consistently phenotypically and cytologically resembled *B. bladhii*. For these reasons, *B. bladhii* was determined to be a compilospecies. This genetically aggressive genome appeared to be gradually consuming closely related species. Estep et al. identified *B. bladhii* sequences derived from all three clades, further supporting the idea that *B. bladhii* is a compilospecies.

Bothriochloa bladhii is a facultative apomict that is commonly a tetraploid, but 5*n*, 6*n*, and 8*n* cytotypes have been reported (J. R. Harlan, 1963). The type specimen (Brown 6184 in the Royal Botanic Gardens, Kew) consists of four different plants representing the great range of variability within the species. *B. bladhii* can be characterized by an inflorescence with an axis as long as the longest racemes, branched lower racemes, broad cauline leaves that are abundant and aromatic, and glumes that may or may not be pitted. The compilospecies' range extends from South Africa, across India, through Indonesia, and down to Australia (J. R. Harlan, 1963).

The characteristics of frequent hybridization with closely related species, morphological variability, and broad geographic distribution seen in *B. bladhii* have been used to describe and identify other compilospecies. Examples of compilospecies include *Armeria villosa* (sea thrift), *Poa pratensis* (Kentucky bluegrass) and *Spiranthes cerna* (nodding lady's tresses) (Aguilar,

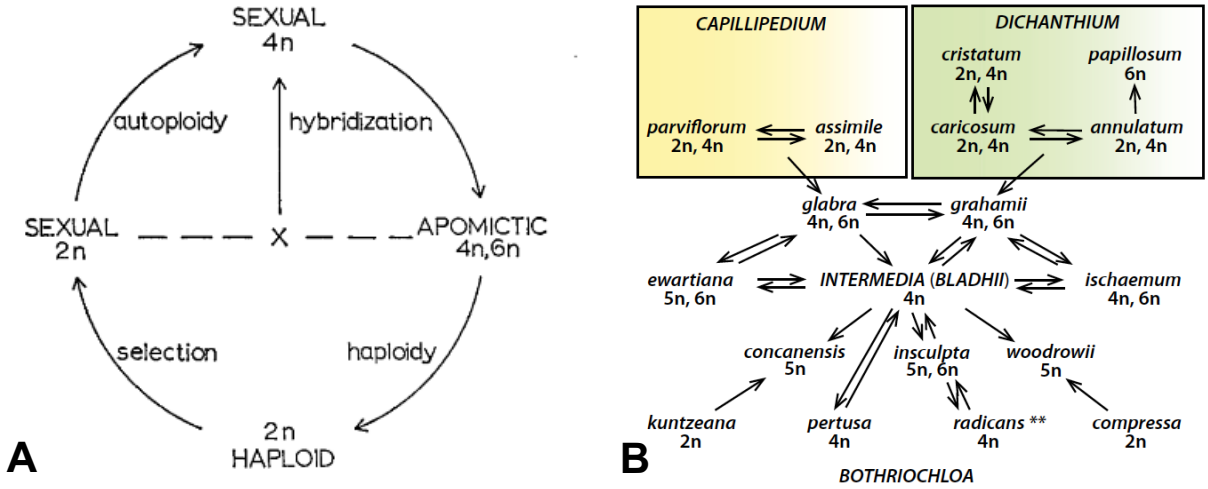

Figure 5. The BCD clade apomictic polyploidy complex structure. A) Diploid-tetraploid-haploid cycle ensure genetic stability and support species boundaries in a dynamic system (de Wet & Harlan, 1970). B) The major species of the BCD clade are all directly or indirectly related. Half arrows indicate the direction of introgression and full arrows show hybrid origin without further introgression (Estep et al., 2014).

Rosselló, & Feliner, 1999; Clausen, 1961; Pace & Cameron, 2017). The genetic aggression of compilospecies has been associated with adaptation to newly disturbed habitats, weediness, and domestication. When introgression is very frequent, a compilospecies may even completely assimilate another species causing it to go extinct (Jack R. Harlan & de Wet, 1963). This "compiling" of genetic material increases genetic variation and may increase fitness of the compilospecies (Anderson, 1953).

Although the mechanism of introgressive hybridization is well described and supported, the compilospecies concept has not been studied in enough depth to be supported with broad consensus. To begin investigation into the compilospecies concept, chromosome counts and cytogenetic behavior in the BCD clade described by Harlan and de Wet must be confirmed and extended with modern molecular tools. This provides a challenge because a consistent and efficient technique for preparing chromosomes for species with many small chromosomes, like in the BCD clade, has not been developed.

Preparing Chromosomes in the BCD Clade

Harlan and de Wet used an acetocarmine chromosome squashing technique to prepare chromosomes of meiotic tissue fixed in Carnoy's II solution (H. R. Chheda, de Wet, J. M. J., Harlan, J. R, 1961; H. R. Chheda & Harlan, 1962; Wet & Borgaonkar, 1963). Anthers were dissected, placed whole onto a slide, and stained. Then, a coverslip was applied to the sample and pressed firmly straight down. This technique uses pressure to squash the anthers and push the meiotic tissue out of the anther. They found this technique challenging and inconsistent, but had no viable alternatives (R. P. Celarier, 1957). Robert P. Celarier (1957), a collaborator of

Harlan and de Wet, described cytological analysis of *B. ischaemum* to be challenging as almost all counts were made at metaphase of meiosis I and the few counts made at diakinesis (prophase I) were poor. It is critical to capture cells between diakinesis and metaphase I, because this is when the chromosomes are condensed, associated in a homologous pair, and naturally well spread. Cells in early stages of prophase (leptotene-zygotene) do not have fully condensed chromosomes. Alternatively, cells in metaphase with chromosomes fully aligned on the metaphase plate, have too many overlapping chromosomes due to high chromosome counts ($2n$ = 20-180) in the BCD clade. R. P. Celarier (1957) also described the size of chromosomes in the BCD clade to be a challenge. Their small size makes them appear as small dots, rather than long thin bars. This results in difficulty discerning individual chromosome structure (banding or knobs), counting overlapping chromosomes, and discriminating between bivalents, paired homologous chromosomes, and univalents, unpaired chromosomes, during the first meiotic division. Therefore, cytogenetic studies with the BCD clade present challenges on multiple fronts: inconsistency in capturing cells in early metaphase, large chromosome number resulting in overlap along the metaphase plate, and small sized chromosomes.

Since Harlan and de Wet studied the BCD clade, numerous advances have been made in plant cytological techniques (Singh, 2017). There are three categories of plant chromosome preparation: squashing, spreading, and dropping (Kirov, Divashuk, Van Laere, Soloviev, & Khrustaleva, 2014). Squashing, the use of pressure to spread cells, is the oldest and most common technique. Although common and simple, it is not useful for species with numerous chromosomes or small chromosomes as the technique provides no mechanism to swell and clear cytoplasm or lengthen chromosomes. Clearing the cytoplasm means to remove it of

proteins, skeletal structure, and any organelles that may interfere with staining. This often done with a weak acid that breaks down or denatures these cellular structures (Ruzin, 1999). The squashing technique provides no mechanism for clearing cytoplasm. Also, squashing helps spread cells to prevent overlap, but doesn't spread chromosomes well. As a result, chromosomes are found on different focal planes and are difficult to visualize with microscopy. Increased pressure does not resolve this issue as too much pressure result in rupture of the cell wall (Kato, 1999).

The spreading technique was developed to overcome the barriers of the cell wall. Mitotic or meiotic tissue is enzymatically digested with cellulase, pectinase, and a variety of other enzymes to break down the cell wall (Ruzin, 1999). This is done directly on the slide in order to increase the concentration of pollen mother cells (PMCs) per slide. The sample is then macerated with the tip of a needle to spread cells (Fukui & Iijima, 1991). This method has been developed for model organisms like maize and rice somatic chromosomes and has been demonstrated to be more suitable for plants with small chromosomes (Kirov et al., 2014).

The previous two techniques lack a mechanism to spread chromosomes. The dropping technique has been adapted to take advantage of steam or shear force to spread chromosomes. Although originally developed for human cells, the dropping technique was further developed in plants to isolate protoplasts through enzymatic digestion (Kirov et al., 2014). Dropping methods prepare a cell suspension via enzymatic digestion and tissue grinding. The cell suspension is dropped from a height of approximately 0.5 m onto an angled slide and the cells are allowed to flow down the slide and air dry. The digesting of the cell wall paired with the force of dropping the suspension is meant to spread the chromosomes and clear the

cytoplasm. This technique is often favored for preparing chromosomes for fluorescence in-situ hybridization (FISH) due to the clearing of cytoplasm. Clearing the cytoplasm increases resolution and decreases interference in FISH (Kuo, Hsu, Yeh, & Chang, 2016). Novel air dry drop methods have been developed in maize and soybean, but they were developed for somatic cells and are not suitable for species with large numbers of chromosomes (Kato, Lamb, & Birchler, 2004).

Although these techniques have been adapted to numerous forms, literature is lacking for a chromosome preparation technique suitable for numerous, small chromosomes. The drop method is the most promising technique for the BCD clade as it aims to swell cells, while simultaneously spreading chromosomes. Recently, an air drop method and a 'SteamDrop' method have been presented as applicable to preparing pachytene and metaphase chromosomes across a broad range of species (Kirov et al., 2014; Kuo et al., 2016). Both methods use a cell suspension that was generated in a microcentrifuge tube. The air drop method enzymatically digests the cell suspension to remove the cell wall. Then, the suspension is dropped onto the slide from a height of up to 0.5 m. The drop generates force that is used to thin the cytoplasm and spread the cells (Kuo et al., 2016). In comparison, the SteamDrop method does not enzymatically digest the cell suspension. Instead, a drop of the suspension is placed on a slide, refixed with a 3:1 ethanol-acetic acid fixative, and then rehydrated with steam. The acetic acid denatures cytoplasmic proteins, which assists in thinning and "clearing" the cytoplasm (Ruzin, 1999). When the cells are rehydrated, the cytoplasm swells and spreads the chromosomes (Kirov et al., 2014). In this study, two chromosome preparation methods adapted from the 'SteamDrop' and modified drop method that are suitable for preparing

chromosomes in the BCD clade are presented. These two methods do not used the same pretreatment, fixation technique, or imaging technique of the air drop and SteamDrop method. Only the cell suspension generation and chromosome preparation technique step is utilized. Recommendations for further cytological work on the BCD clade based on these experiences are also suggested.

Methods

Plant Material Collection and Fixation

Grasses were grown from USDA and Australian Tropical Crops and Forage Collection seeds and are maintained in the Appalachian State Biology Greenhouse (Table 1). Inflorescences were collected while the grasses were in very early booting stage of inflorescence development. During booting, the inflorescence is fully developed, but still completely enclosed within the leaf sheath. This stage was identified by a flag leaf standing erect above a swollen leaf sheath (Figure 6).

Inflorescences were separated from the leaf sheath and immediately placed in 2 mM 8-hydroxyquinoline for 3 hours at 4°C. 8-hydroxyquinoline inhibits spindle fiber formation causing the cell to arrest in metaphase (Ekong N. J., 2013). Samples were then transferred to either Carnoy's II solution (6:3:1 100% ethanol, chloroform, glacial acetic acid) or 4% paraformaldehyde for a minimum of 24 hours at 4°C. Samples fixed in Carnoy's II solution were then transferred to 70% ethanol and stored in a -20°C until use.

Screening Inflorescences

Inflorescences were screened by rame to determine their stage of microsporogensis. A single pedicellate spikelet was analyzed per rame. The anthers were dissected from the floret and stained with 2-3 drops of 2% acteo-orcein for 10 minutes. The anthers were then squashed by placing a coverslip on the sample and pressing firmly straight down to apply an even pressure across the sample. The slide was then imaged using a BA410E Binocular 50W microscope and imaged with a Meiji Techno HD1500T camera. Spikelets in the correct developmental stage from the same inflorescence were prepared for further examination by an air drop or modified SteamDrop chromosome preparation (Kuo, 2016; Kirov, 2014).

Chromosome Preparation: Air Drop

A rame in the correct stage of meiosis was dissected (10-15 pediculate spikelets) and the anthers were transferred to 0.1 M citrate buffer (pH 4.8). The anthers were lightly ground

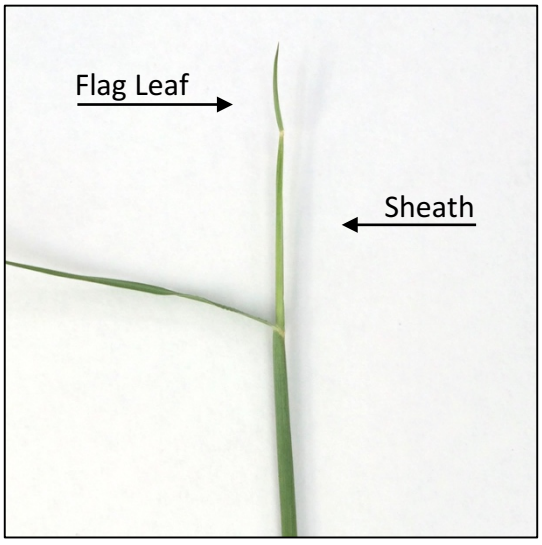

Figure 6. An example of a flagging leaf from a *Bothriochloa* species when an immature inflorescence was collected.

with a micropestle to break the anthers and release the pollen mother cells (PMCs) into solution. The cell suspension was incubated with a digestion enzyme mixture (4 mg cellulose, 4 mg pectinase, and 20 μL of Triton X-100 in 400 μL citrate buffer) for 10 minutes (Ruzin, 1999). The cell suspension was washed twice with distilled water, re-fixed with three washes of Carnoy's II solution, and then resuspended in 200 μL Carnoy's II solution.

Table 1. A list of species used to develop and test the described method including their USDA and Australian Tropical Crops and Forage Collection accession numbers.

Species	Accession Number
Bothriochloa pertusa	PI 301642
Bothriochloa spp.	PI 502298 02
Bothriochloa spp.	PI 502299 01
Bothriochloa spp.	PI 531224 01
Bothriochloa spp.	PI 531225 01
Bothriochloa spp.	PI 531226 01
Bothriochloa spp.	PI 531227 02
Capillipedium parviflorum	PI 201763 O 711
Capillipedium parviflorum	PI 301751 A 9403
Capillipedium spicigerum	PI 301773 A 9428
Capillipedium venustum	PI 301731 A 2655
Dichanthium annulatum	PI 219638
Dichanthium annulatum	PI 301868
Dichanthium foveolatum	PI 302076
Dichanthium sericeum	AusTRCF323610
Dichanthium sericeum	AusTRCF323617
Dichanthium sericeum	AusTRCF323619
Dichanthium sericeum	AusTRCF323620
Dichanthium sericeum	AusTRCF323621
Dichanthium sericeum	AusTRCF323624
Dichanthium spp.	AusTRCF104671
Dichanthium spp.	AusTRCF104817
Dichanthium spp.	AusTRCF104837
Dichanthium spp.	AusTRCF106120
Dichanthium spp.	AusTRCF118686
Dichanthium spp.	AusTRCF319659

An air drop technique modified from Kuo (2016) was used to evenly spread chromosomes on a glass microscope slide. The cell suspension was dropped from a height of 3 inches onto a slide inclined 30°. The suspension was allowed to flow down the inclined plane to spread the cells and the slide was left to air dry.

Chromosome Preparation: Modified SteamDrop

Alternatively, some rames previously determined to be in the correct stage of meiosis were prepared using a modified SteamDrop technique. A rame was dissected (10-15 florets) and the anthers were placed in distilled water. The anthers were lightly ground with a micropestle and washed twice in 96% ethanol. The cell suspension was dropped onto a slide and the ethanol evaporated until it was only present in a thin layer over the cells (Kirov, 2014). This stage was easy to identify as the thin layer of liquid gave the slide a granular appearance. A drop of 3:1 ethanol-acetic acid fixative was then dropped onto the sample. When this fixative formed a thin layer of liquid over the cells, the slide was held over a water bath of 55 °C for 3-5 seconds to rehydrate the cells with steam. The slide was immediately dried with air flow.

Staining

Slides prepared using either of the two methods were stained with aceto-orcein or DAPI (0.5 µg/mL) for 5 to 10 minutes (Ruzin, 1999). These dyes are used to visualize chromosomes and chromatin. Slides stained with aceto-orcein were drained to removed excess stain. Slides stained with DAPI were kept in complete darkness during incubation and rinsed lightly with distilled water to remove excess stain after staining was complete. Lastly, a drop of mounting

medium was placed on the DAPI-stained sample. A coverslip was gently placed on all slides to complete preparation.

Microscopy and Imaging

Slides were initially examined and imaged under a BA410E Binocular 50W microscope and Meiji Techno HD1500 camera. High quality slides were further examined with an Olympus IX81/DP71 in the Appalachian State William C. and Ruth Ann Dewel Microscopy Facility. Image processing and optimization were preformed using either Photoshop (Adobe Inc., San Jose, California, USA) or ImageJ (Schneier, 2012).

Results

Collection of Flower Buds and Examination of Chromosome Stage

Collected inflorescences were initially pretreated with 8-hydroxyquinoline (HQ) for 3 hours. Inflorescences not pretreated contained only fully developed pollen and no pollen mother cells (PMCs) were observed (Figure 7A). When treated with HQ, inflorescences with spikelets 3-4 mm in size were found to contain PMCs where chromosomes are visible (Figure 7B). Spikelets less than 3 mm in size showed no development of PMCs indicating those inflorescences are underdeveloped (Figure 7C). Only 5% (1 in 20) of the inflorescences in the ideal size range were observed to be in late prophase or metaphase with visible chromosomes.

After pretreatment, inflorescences were transferred to either Carnoy's II solution or 4% paraformaldehyde for fixation. Inflorescences fixed in Carnoy's II solution were later transferred to 70% ethanol for storage. Long-term storage in 4% paraformaldehyde resulted in thickening

of the cytoplasm and unusable tissue (Figure 8). Pollen mother cells appeared to have cloudy cytoplasm that drastically decreased resolution (Figure 8A). Tissue fixed in Carnoy's II solution had clear cytoplasm and condensed chromosomes were clearly visible (Figure 8B). Storage in Carnoy's II solution for longer than a month was observed to cause rigidity of PMCs. This can make PMCs more susceptible to maceration in later steps. Tissue was stored in 70% ethanol to maintain cellular integrity.

Preparation of Chromosomes Using an Air Drop Method

The cell suspension generated from a single inflorescence was used to prepare 9-10 slides with similar results. A micropestle was used to break the PMCs from the anthers into solution. Enzymatic digestion with cellulase and pectinase was then used to break down the cell wall. This allowed for more flexibility in cell shape and aided in digesting cytoplasmic components that could reduce clarity. Triton X, a detergent that solubilizes phospholipid membranes, was included in the digestion cocktail and allowed faster staining as aceto-orcein can easily cross a permeable cell membrane (Ruzin, 1999). Digestion of the cell wall and

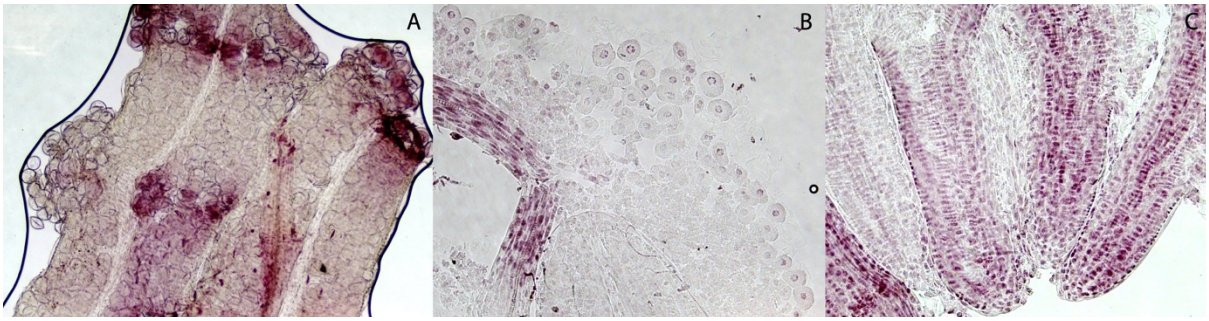

Figure 7. Stages of pollen development across spikelet size. A) Spikelets greater than 4 mm contain fully developed pollen B) Spikelets between 3-4 mm contain pollen mother cells undergoing meiosis C) Spikelets less than 3 mm show no development of meiotic tissue.

cytoplasm combined with a more permeable membrane resulted in visible, condensed chromosomes and images with high contrast (Figure 9A-C).

Digestion of the cell wall improves clarity, but also causes fragility of the PMCs. This resulted in maceration of PMCs during washing and refixation steps (Figure 9D). An estimated 90% of PMCs were lost due to maceration. Cells that remained intact were surrounded by fragments of macerated PMCs (Figure 9A-C) and often had a distorted shape (Figure 9C). Although digestion did increase contrast of chromosomes against the cytoplasm compared to squashes used in the screening step (Figure 7B), chromosomes still remained overlapped and difficult to resolve, therefore impossible to count even under high magnification (Figure 8B). Dropping the cell suspension onto the slide provided enough force to spread PMCs but not enough to stretch individual cells and spread chromosomes. It also did not clear the cytoplasm

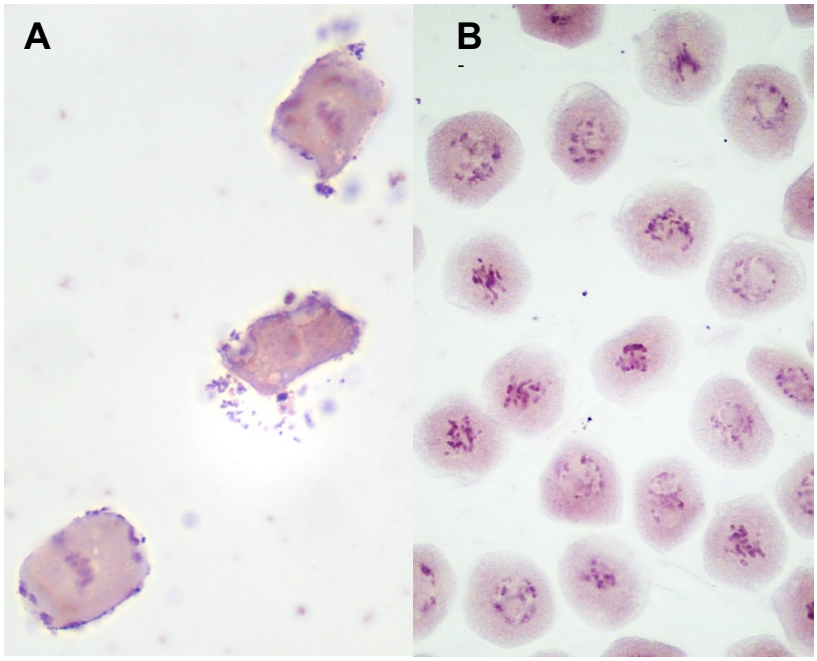

Figure 8. Comparison of cellular structure after long-term storage in A) 4% paraformaldehyde B) Carnoy's II solution.

causing cells to have a gritty, textured appearance (Figure 9A). This further decreased resolution of individual chromosomes. Meiotic spreads in the BCD clade could not be successfully prepared using the air drop method.

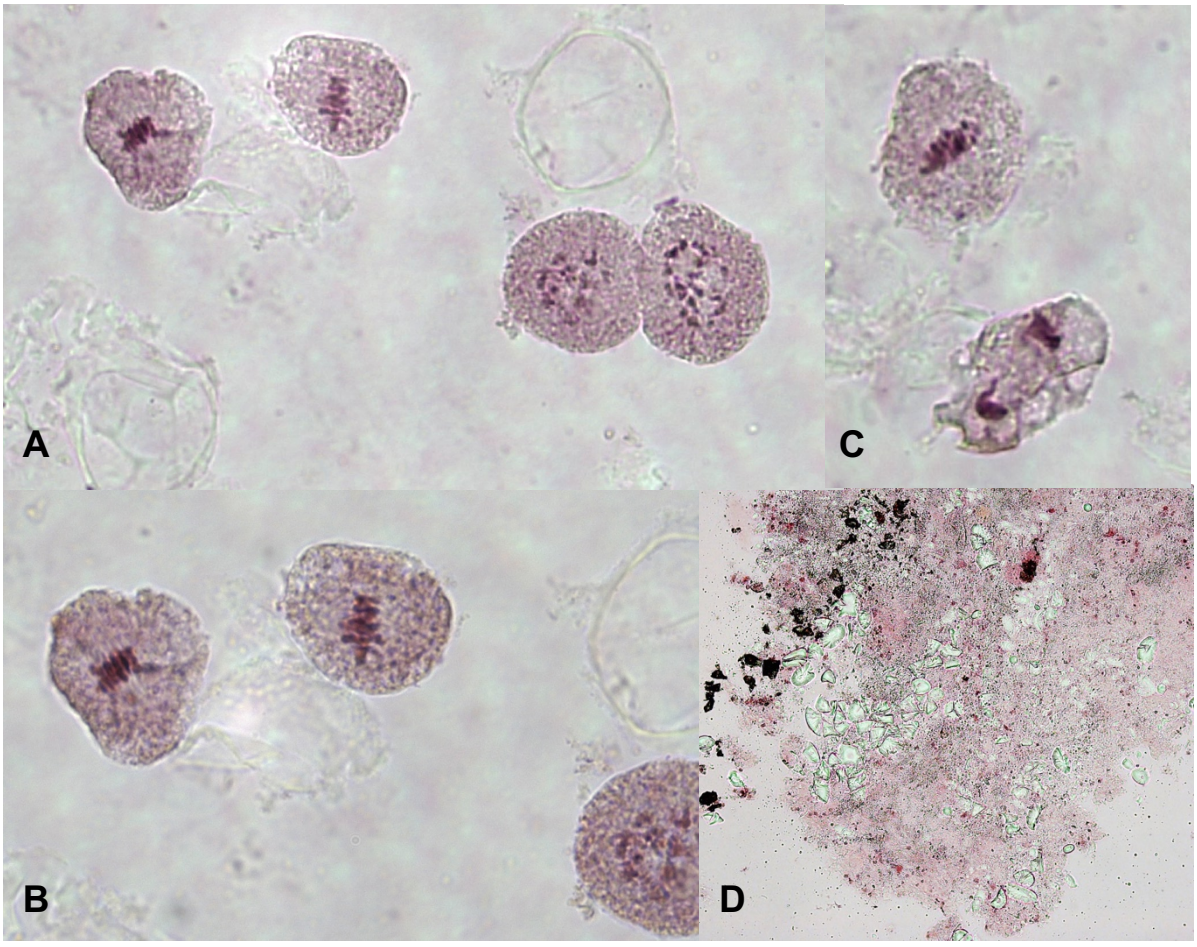

Figure 9. Pollen mother cells from a *Bothriochloa* species prepared using an air drop method. A) Two PMCs in prophase (bottom right cells) and two PMCs in metaphase (upper left cells) under low magnification (40X) surrounded by debris from macerated cells. B) PMC in prophase (bottom right) and two PMCs in metaphase (left) under high magnification (100X oil). C) A PMC in metaphase (left) and a distorted PMC in anaphase (right) (40X). D) Fragmented PMCs.

Preparation of Chromosomes Using a Modified SteamDrop Method

Similar to the air drop method, the cell suspension generated from a single inflorescence was used to prepare 6-7 slides of consistent quality. Anthers were ground lightly with a micropestle, but were not enzymatically digested because steam should penetrate the cell wall and membrane. The lack of enzymatic digestion resulted in no noticeable maceration of PMCs. drop of the cell suspension was placed on the slide, re-fixed, and rehydrated by steam. Compared to an aceto-orcein squash, where chromosomes are overlapping and difficult to resolve (Figure 10A), cells prepared with the modified SteamDrop method experienced swelling of the cytoplasm that spread chromosomes across the same focal plane with minimal overlap (Figure 10B). Minor cell shrinkage was observed using this method but this did not prevent the spreading of chromosomes (Figure 10B). Elongation of chromosomes was also observed in samples prepared by the modified SteamDrop method (Figure 10C). The SteamDrop method successfully spreads chromosomes and elongates chromosomes but still lacks an efficientpretreatment that will arrest cells in diakinesis and metaphase. For these reasons, chromosomes still cannot be effectively counted using the SteamDrop preparation technique.

Discussion

When developing a method to prepare chromosomes in a specific group, each step must be adjusted to account for secondary interactions and unexpected conditions. In the

BCD clade, these special circumstances include, but are not limited to, summer flowering, fragile PMCs, small chromosomes, and large number of chromosomes. These factors must be A accounted for when choosing a pretreatment, fixative, and chromosome preparation technique.

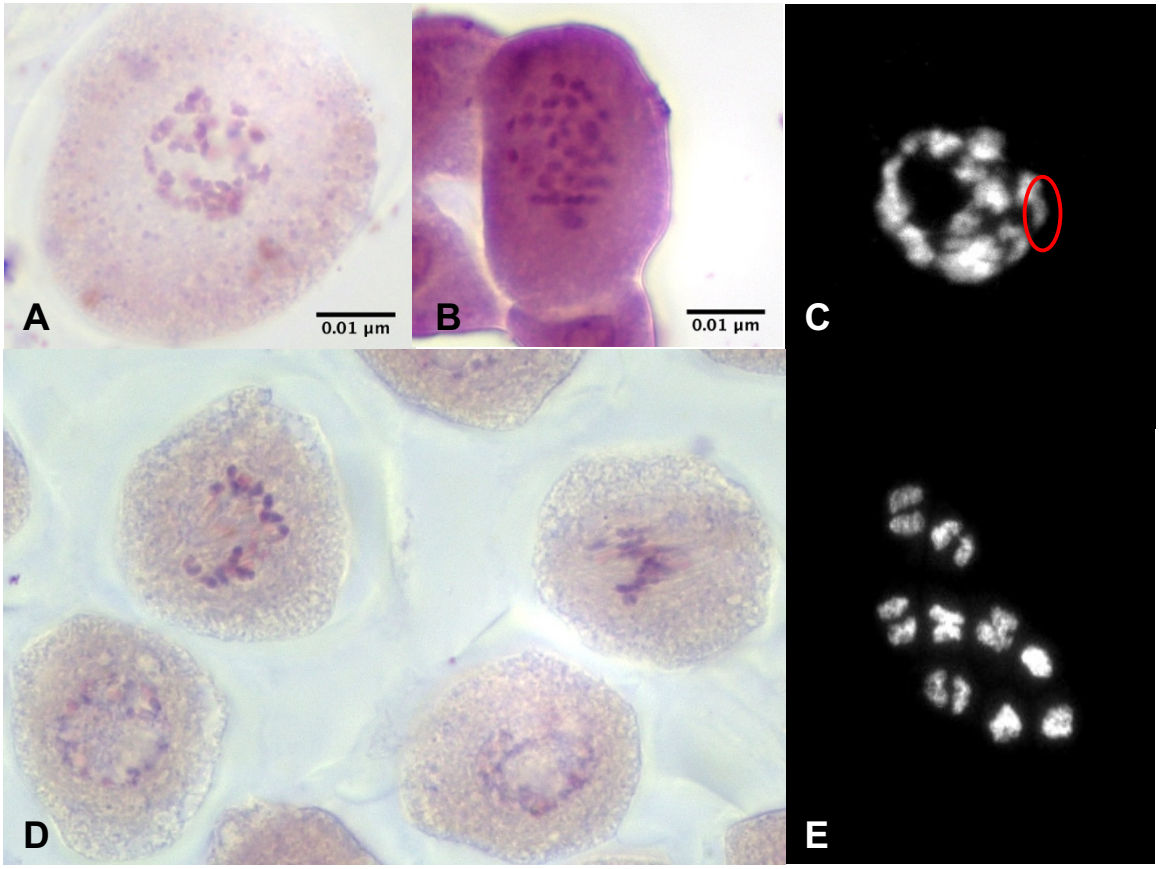

Figure 10. Pollen mother cells from a *Dichanthium* species. A) PMC prepared using an aceto-orcein squash. Chromosome are overlapping and on different focal planes (100X oil). Using a modified SteamDrop method (B-E), chromosomes are well spread and elongated allowing better resolution. B) PMC with well-spread chromosomes on the same focal plane. Minor cell shrinkage is observed compared to the squashed PMC (A) (100X oil). C) PMC stained with DAPI an imaged with a confocal microscope to show higher resolution of chromosome structure (40X oil). Red circle designates a single chromosome. D) 4 PMCs in prophase I (lower left and lower right), metaphase I (upper right), and anaphase I (upper left) depicting the large range of meiotic stages prepared on a single slide that makes preparing chromosomes in the BCD clade challenging (40X). E) PMC in anaphase stained with DAPI (20X).

Improvements in Collection and Fixation

Inflorescences were treated with HQ immediately after collection to ensure cells were fixed in the phase of meiosis they were in upon collection. It is preferable to work in meiotic tissue over mitotic tissue when dealing with species with high chromosome counts because gametes typically have half the chromosomes of somatic tissue. Like many of the panacoid grasses, the BCD clade flowers in the summer and fall with the majority of inflorescences collected from August to October. Therefore, tissue samples have to be collected on a mass scale and often stored for several months. An efficient and effective pretreatment-fixation protocol is necessary to ensure collected samples are arrested in the correct stage of meiosis and properly preserved.

Pretreatment with HQ helps to ensure the cells are fixed during the short time window of late prophase to metaphase. 8-hydroxyquinoline inhibits spindle fiber formation causing the cell to arrest in metaphase (Ekong N. J., 2013). Therefore, any cells entering metaphase or undergoing anaphase will not be able to complete these stages. If the spindle fibers are not inhibited fast enough (slow infiltration of HQ), PMCs can move through anaphase I and cytokinesis completing the first round of meiosis. Many of the PMCs observed during inflorescences screening were in metaphase or anaphase, suggesting that HQ was not inhibiting spindle fibers fast enough in these experiments (Figure 4D & E). The PMC in Figure 4C has condensed chromosomes but also many overlapping globular structures that are partially condensed chromosomes, making accurate counts difficult. The inability to arrest cells in a stage where chromosomes are fully condensed and not-overlapped remains a challenge for preparing chromosomes in the BCD clade.

An alternative or co-pretreatment may be required to arrest the majority of cells in the correct stage. If we assume the HQ is not penetrating the cell wall/membrane fast enough, a solution might be to treat the tissue with liquid nitrogen to generate many openings within the cell. Abbassi (2014) demonstrated liquid nitrogen's ability to increase permeability of the cell wall in algae through cracking resulting from thawing. Inflorescences dipped in liquid nitrogen would have more permeable cell walls that would allow faster penetration of HQ and Carnoy's solution. Increasing the rate of fixation and decrease the number of cells that move forward through prophase or metaphase. This approach may create artefacts that are currently unknown as there is little to no literature for using liquid nitrogen as a pretreatment for chromosome preparation. Liquid nitrogen is typically used to fix cells onto slides before staining (Fukui, 1996). Finding a method to speed the activity of HQ should be prioritized in future experiments.

The second aspect of chromosome preparation analyzed in the BCD clade was fixation. A good fixative digests all cellular components except chromosomes, preserves tissue structure with no osmotic swelling, and doesn't interfere with staining properties of tissues (Ruzin, 1999). In this method, tissue was fixed in either Carnoy's II solution or 4% paraformaldehyde. Paraformaldehyde fixes tissue through chemical crosslinking of protoplasmic components and the chemical crosslinks are retained in tissues after processing (Ruzin, 1999). As a result, the PMC observed showed cytoplasm thickening, causing low resolution and alteration of cell structure (Figure 3A). Long-term storage in 4% paraformaldehyde further resulted in cellular degradation and unusable tissue. The acetic acid in Carnoy's II solution denatures cytoplasmic proteins and the alcohol transforms protoplasm into an artificially interconnected network.

Transferring the inflorescences to 70% ethanol for storage prevented over-digestion of the cytoplasm while maintaining the artificial network created by the alcohol (Ruzin, 1999).

Benefits of Generating a Cell Suspension

Dropping techniques differ from squashing and spreading techniques largely due to the generation of a cell suspension. Squashing and spreading techniques process an entire inflorescence directly on a single slide. This can result in a thick tissue sample that shortens the focal distance during imaging, therefore decreasing resolution. Cell suspensions slightly dilute the PMC to prevent overlap and create ease in spreading cells. Processing a whole inflorescence directly on the slide also means you can only generate one slide per inflorescence. If an error occurs during preparation in a squashing or spreading method, an entire sample would be lost. Generation of a cell suspension increases resolution and permits replication.

Disadvantages of the Air Drop Method

Originally developed to overcome the rigidity of the cell wall in chromosome preparation, dropping techniques classically rely on enzymatic digestion for the removal of cell walls and permeabilization of the cell membrane. In the BCD clade, enzymatic digestion makes PMCs incredibly fragile and easily macerated in subsequent washing steps (Figure 4D). When PMCs survived intact, cytoplasm was not observably cleared in the manner described by Kuo et al. (2016). 'Bombardment' of the cells onto the slides using the described method was supposed to stretch or burst the cells, thinning or clearing the cytoplasm, which should increase resolution of the chromosomes. Clearing cytoplasm is particularly important because

fluorescent in-situ hybridization is most successful on samples with minimal cytoplasmic interference (Kuo et al., 2016). Overall, the air drop technique is not successful in the BCD clade due to fragility of PMCs after enzymatic digestion and failure to clear cytoplasm resulting in overall low resolution.

Advantages of the Modified SteamDrop Method

The modified SteamDrop technique swells cells and spread chromosomes across the same focal plane through rapid rehydration of cells (Kirov et al., 2014). The use of steam induces rapid cell wall hydrolysis, cytoplasm rehydration and swelling, and chromosome movement; all of which were observed in BCD clade samples (Figure 5A-B). Once the cell suspension is initially dropped on the slide and the ethanol evaporates, the sample is re-fixed in a fixative composed of ethanol and acetic acid. The reintroduction of alcohol strengthens the cytoplasmic network formed during the initial fixation, while the acetic acid clears the cytoplasm of any artefacts that may have formed during processing (Ruzin, 1999). This process of dropping and re-fixing maintains cellular integrity and preps the cells for the shock of rapid rehydration.

Introduction of steam when the fixative has almost evaporated from the slide and is in a thin layer over the cells resulted in swelling of the cytoplasm (Figure 10B). Swelling is not thought to be a result of membrane-mediated osmosis, but instead caused by fixation-induced alterations to the cytoskeleton (Claussen et al., 2002). Steam introduces water equally across the slide surface allowing even cytoplasm rehydration and swelling across the cell (Henegariu et al., 2001; Kirov et al., 2014). The even expansion of the cytoplasm pulled the chromosomes

along to create a spread across a single focal plane (Figure 10B). If evaporation of fixative occurs faster than rehydration of the cell, cell shrinkage can occur, preventing maximum chromosome spreading. Increasing the temperature of the water bath used for steam can prevent the minor cell shrinkage that was observed (Figure 10B). Low humidity results in the ethanol component of the fixative evaporating faster than the cell is rehydrated, resulting in a high concentration of acetic acid that can over digest the cytoplasm and prevent swelling (Kirov et al., 2014; Sharma & Sharma, 2001). In future uses of this method, it is recommended that either the concentration of acetic acid in the fixative is lowered or that the temperature of the water bath used to generate steam is increased to avoid cell shrinkage.

The combination of pressure from the evaporating fixative and rapid rehydration can also result in elongation of chromosomes (Henegariu et al., 2001). This was observed in several instances (Figure 10C). What causes chromosome stretching is still not fully understood, but Kirov et al. (2014) showed moderate humidity in steam treatments results in chromosome spreading and larger, less stretched chromosomes. This suggested that modifying the temperature of steam treatments could influence the amount of stretching. Distorted chromosomes will affect analyses such as genome mapping and should be avoided in these preparations (Claussen et al., 2002; Henegariu et al., 2001).

Cytogenetic studies on the BCD clade are difficult because of the large number of very small chromosomes. The modified SteamDrop method overcomes two of the three major barriers of preparing chromosomes in the BCD clade. This approach was able to spread the large number of chromosomes with minimum overlap and should allow for accurate chromosome counts in future studies. The protocol also slightly elongated the small sized

chromosomes allowing for the potential description of structures like chromosome knobs or specific banding patterns. Ergo, the modified SteamDrop has been demonstrated to be an effective method to visualize chromosomes in the BCD clade.

The last barrier to easy preparation of chromosomes in the BCD clade is the inconsistency of capturing cells in late prophase or early metaphase. Observations using HQ as a pretreatment suggest cells are not being arrested quickly enough. Methods to facilitate infiltration of HQ must be examined further to overcome this roadblock.

Further Application

In order to confirm chromosome counts and cytogenetic behavior described by Harlan and de Wet, this method will be applied to obtain chromosome counts, describe chromosome association (univalent, bivalents, etc.), and record other chromosome and meiotic abnormalities during future studies. Modern molecular tools, such as fluorescent in-situ hybridization, will be applied to karyotype members of the BCD clade and observe chromosome behavior in subgenomes with a focus on hybrids and genome dominance.

Few geneticists or plant breeders deliberately take advantage of polyploidy and interspecific hybridization for crop improvement (Mason & Batley, 2015). The process of domestication reduces genetic diversity and many crops have experience significant losses of allelic diversity. Introgressive hybridization may be beneficial in introducing desirable traits from wild relatives back into domesticated crops. Studying introgressive hybridization and genome dominance in a compilospecies, like the one found in the BCD clade, may help us better understand how traits can be introduced into polyploidy cereals.

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